

**DEVELOPMENT AND ASSESSMENT OF AVIAN AND OVINE
ANTIVENOMS FOR EUROPEAN VIPER VENOMS**

By

KENNETH LOUIS HARRISON

BSc.(Hons) Applied Biology
MSc.Molecular Pathology

Department of Chemical Pathology,
St Bartholomew's & The Royal London School of Medicine & Dentistry,
London, UK.

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**BROKEN TEXT AND SOME
POOR QUALITY IMAGES IN
ORIGINAL THESIS.**

ABSTRACT

This research was undertaken in order to design techniques and processes that enable the manufacture of effective antivenoms.

To prepare a broad specificity antivenom for European vipers from chicken yolk it was first necessary to develop a simple effective method to extract avian immunoglobulin (IgY). A specific fluoroimmunoassay was developed to monitor IgY recovery and serum IgY levels in immunised hens.

The most promising extraction methods from the literature were compared using a triglyceride kit to monitor lipoprotein removed and SDS-PAGE and ELISA to monitor purity and activity respectively. Caprylic acid followed by ammonium sulphate proved the best method. Unfortunately only low levels of specific IgY were achieved and it was necessary to include an affinity purification step to demonstrate their effectiveness in an ED₅₀ test.

Pepsin, papain and trypsin all produced Fab' fragments from IgY but only pepsin digested the resultant Fc fragments. Pepsin could also digest other proteins in egg yolk, thereby avoiding the need to salt fractionate IgY prior to its digestion with a consequent improvement in the recovery of Fab'.

A small scale affinity purification (SSAP) assay was developed, characterised and used to determine specific antibody levels in ovine antisera. Small doses (15µg) of venom produced significant specific levels but larger doses produced a better response and were used to produce antivenom. Binding studies with SSAP demonstrated a high concentration of specific antibodies in *V.latastei* antisera that bind to components in the venoms of other European vipers. A specific ovine F(ab')₂-based *V.latastei* antivenom approximately twice as potent as the antivenom used currently in Spain was prepared from the ovine antisera.

Evidence is presented that SSAP should supersede manual ELISA for assessing specific antibody levels in antisera.

No major gain in recovery and purity resulted from processing whole blood rather than serum for preparing antivenom.

ABBREVIATIONS

AR	: Analytical recovery
CNBR	: Cyanogen bromide
CV	: Coefficient of variation
ED₅₀	: Median effective dose of antivenom
EDTA	: Ethylenediamine tetra-acetic acid
ELISA	: Enzyme linked immunosorbent assay
FITC	: Fluorescein isothiocyanate
HB	: Histidine base
HBS	: Histidine buffered saline
HRP	: Horseradish peroxidase
IgG	: Immunoglobulin G
i.m.	: Intramuscular
i.v.	: Intravenous
IgY	: Immunoglobulin Y
kDa	: Kilodalton
LD₅₀	: Median lethal dose of venom
NSB	: Non specific binding
OD	: Optical density
OPD	: O-phenylenediamine dihydrochloride
PBS	: Phosphate buffered saline
PEG	: Polyethylene glycol
SDS - PAGE	: Sodium doedecyl sulphate – polyacrylamide gel electrophoresis
SSAP	: Small scale affinity purification
TRIS	: Tris(hydroxymethyl) aminomethane

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Immunotherapy

1.1.1 History and general introduction

Inoculation with crusts derived from the pustules of favourable cases of smallpox was practiced widely by many cultures in Asia, Africa and even in the rural areas of western Europe. Voltaire speculated that inoculation might have originated with the Circassians to protect the beauty of their daughters, whom they could not sell pock-marked into the harems of the Ottoman Empire (Silverstein, 1984).

It was in this context of three quarters of a century of smallpox inoculation that Edward Jenner published, in 1798, his report on a safer and more efficacious vaccine against small pox, derived from cowpox pustules. Jenner appears never to have speculated on why his vaccine caused immunity, perhaps influenced by the earlier advice of his teacher John Hunter: “ Why think ? Why not try the experiment.”

Louis Pasteur led the battle to convince the scientific community of the validity of the germ theory. His report in 1880 that prophylactic immunisation against chicken cholera was possible, marked the start of scientific immunology (Silverstein, 1984). As new pathogens were identified, each was the subject of intensive investigation to develop a suitable vaccine. In 1888 Emile Roux and Alexandre Yersin demonstrated that a soluble toxin could be isolated from the supernatants of cultures of the diphtheria organism and in 1890 Von Berhing and Kitasato demonstrated that animals immunised with diphtheria and tetanus toxoid produced something in their blood that could neutralize the toxin. Antitoxic sera from experimental animals were quickly tested in infected children and were shown to produce remarkable and rapid cures. The substance that acted against the toxin was called anti-toxin, and soon the more general and non-committal term antibody was used to describe the new class of substances.

In 1887 Henry Sewall immunized pigeons against rattlesnake venom by injecting them with very low and progressively increasing doses of the venom.

Calmette, who was a physician, had acquired experience in scientific experimentation while training in microbiology at the laboratory of Emile Roux in the Institut Pasteur in Paris. He had investigated the physiological effects of snake venoms in several animals and postulated an analogy between the components of snake venoms and bacterial toxins. He published his investigations using Cobra venom, which form the basis of antivenom therapy, in the *Annales de l'Institut Pasteur* (1894). Phisalix and Bertand (1894) presented their own observations on the antitoxic properties of the blood of animals immunized against viper venom in the same year and in the same journal.

Although antivenom therapy was thus independently discovered by two groups, the general consensus is that Albert Calmette is the true father of antivenoms.

1.1.2 Definitions of immunity (McFerran, 1995)

1.1.2.1 Active

Immunity that arises when the body's own cells produce, and remain able to produce, appropriate antibodies following an attack of an infectious disease or deliberate stimulation.

1.1.2.2 Passive

The immunity which results when an animal is injected with immunoglobulins obtained by the active immunisation of another animal.

1.1.2.3 Natural

The immunity which a young animal acquires from its mother's immunoglobulins.

1.2 Immunoglobulins

1.2.1 Why they have evolved (Roitt, 1988)

When microbes penetrate the body, two main defensive operations come into play (Figure 1.1). The first in evolutionary terms, is referred to as the "innate" system because it is not affected by prior contact with the infectious agent. It includes phagocytosis, the process by

which special cells engulf a foreign organism and enclose it within their cytoplasm. Before phagocytosis can occur the phagocyte must home in on the microbe, adhere to it and undergo membrane activation which initiates engulfment. Some bacteria produce chemical substances which attract phagocytes, some microbes adhere to the phagocyte's surface and some spontaneously provide the appropriate membrane initiation signal. However many microbes have evolved mechanisms to avoid phagocytosis and, to counteract this, the body evolved the second component of "innate" protection namely the complement system. This comprises a complex series of proteins which form one of several triggered enzyme systems found in plasma and characteristically produce a rapid, highly amplified response to a stimulus, involving a cascade whereby the product of one reaction is the enzymic catalyst of the next. Complement has a range of biological functions that include adherence reactions to microbes and the creation of lesions in their membranes.

Again microbes have evolved strategies to evade such defences. For example some bacteria have shaped their exteriors to avoid complement activation completely. Thus the body needed to "devise" a second system of defence mechanisms individually tailored to every type of microbe encountered. The evolutionary process came up with a solution that involved a molecule intrinsically capable both of activating the complement system and of stimulating phagocytic cells, as well as adhering specifically to the offending microbe.

From the onset such molecules had one region concerned in communicating with and activating complement and phagocytosis (the effector component) and another devoted to binding specifically to an individual microbe (the binding component). While the effector component can be common, the body has to be able to make millions of binding components with different recognition sites. The recognition and effector components make up the molecules we recognize as antibodies and the humoral immune response is the mechanism whereby the body synthesises large numbers of appropriate antibodies in the event of microbial infection. This is part of the second major defensive mechanism.

PHAGOCYTOTIC CELLS KILL MICRO-ORGANISMS



COMPLEMENT FACILITATES PHAGOCYTOSIS



ADDITIONAL MECHANISMS PROVIDE A SECOND DEFENSIVE STRATEGY

- (a) Acute phase proteins
- (b) Interferons
- (c) Natural killer cells
- (d) Eosinophils



The body needed to devise defence mechanisms designed individually to each organism involved



ANTIBODY THE SPECIFIC ADAPTOR

Evolutionary processes have shaped an adaptor molecule intrinsically capable of activating complement, stimulating phagocytic cells, and binding to the offending microbe. To do this the body has to be able to make myriad adaptors with different recognition sites

Figure 1.1 Summary of why antibodies have evolved. Adapted from Essential Immunology (Roitt, 1988)

1.2.2 Types and sources (Landon and Chard, 1995)

Antibody preparations can be discussed according to their source and whether they are monoclonal or polyclonal (Table 1.1). Antibodies used for therapeutic purposes are usually of the IgG class because of their high circulating concentrations, excellent affinity and stability. Human immunoglobulins are often used to treat infectious diseases, especially in immunocompromised patients. Unrefined human plasma, collected by venepuncture or plasmaphoresis, can be infused with little risk of inducing side effects. However, supplies of human plasma are limited; it is unethical to immunize subjects with infectious agents, tumour products or drugs; the affinities and titres of specific antibodies in human plasma tend to be low and there is a risk of transferring infections such as hepatitis.

These problems are all overcome by the use of other mammalian immunoglobulins, but are then replaced by the more serious risk of causing an allergic response. Therapy usually demands large amounts of antibody and polyclonal immunoglobulins for this purpose are normally produced in sheep or horses and administered in the form of purified concentrates. Avian antibodies, derived from hens' eggs, may well prove to be ideal for oral use, but the risk of causing severe allergic reactions may prohibit their systemic administration.

1.2.3 Structure and function

1.2.3.1 Overview (Huber, 1984; Roitt, 1988; Bentley, 1994)

The basic structure of all immunoglobulin (Ig) molecules (Figure 1.2) comprises two identical light (L) chains and two identical heavy (H) chains linked together by disulphide bonds. There are two different classes (isotypes) of light chains, λ and κ . Heavy chains, by contrast, have five different isotypes that divide the immunoglobulins into different functional classes, namely, IgG, IgM, IgA, IgD, and IgE, each with different effector properties. Each class of heavy chain can combine with either of the two different classes of light chain.

Immunoglobulins of class G (IgG), which is the major type of immunoglobulin in normal

Polyclonal antibodies

Intact:	
Human IgG	Given as plasma or an immunoglobulin concentrate: The latter may be enriched with IgM
Animal IgG	Usually an equine immunoglobulin concentrate
IgA	Given orally and usually from bovine milk
Avian IgY	Given orally as whole egg, egg yolk or purified IgY
Fragments:	
F(ab') ₂	Usually equine
Fab	Usually ovine
Fv	

Monoclonal antibodies

Normal or activated human B lymphocytes	
Activated primate B lymphocytes	
Human myeloma cells	
Hybridomas	Homogeneous, usually murine: murine Heterogeneous, usually human: murine
Quadromas	
Transfectomas	
Transgenic animals	

Table 1.1 A classification of types and sources of antibodies for therapeutic use. (Landon and Chard, 1995)

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human serum, are monomers of the basic unit. Each chain of an IgG molecule is divided into domains of about 110 amino acid residues with the light chains having two such domains, and the heavy chains four. The amino-terminal domain of each polypeptide chain is highly variable whereas the remaining domains have constant sequences for each class. A light chain is thus built up from one amino-terminal variable domain (V_L) and one carboxy-terminal constant domain (C_L) while a heavy chain has one amino-terminal variable domain (V_H), followed by three constant domains (C_{H1} , C_{H2} , and C_{H3}).

The variable domains are not uniformly variable throughout their lengths. In particular, three small regions show much more variability than the rest of the chain and are called the hypervariable or complementarity determining regions, CDR1-CDR3. They vary both in size and in sequence between different immunoglobulins and are the regions that determine the specificity of the antigen -antibody interactions.

Each Ig molecule is bifunctional, with one part binding to the target antigen and the other mediating the effector functions. The latter include: (i) elimination or neutralization of antigen (as for example with toxins); (ii) binding of antigen to tissues by interaction with Fc to assist phagocytosis, antigen processing or the release of antimicrobial factors; and (iii) fixation of complement.

1.2.3.2 Enzymatic cleavage

1.2.3.2.1 Pepsin (Samloff, 1971)

Pepsin is an acidic protease (MW35,000Da: Isoelectric point pH 1), and the principal proteolytic enzyme of vertebrate gastric juice.

There are several minor pepsins designated B,C and D; the major component is A to which the following information applies. Pepsin is derived from its precursor pepsinogen by autoactivation at acidic pH. It adopts a bilobal structure with the first 171 amino acids forming one lobe and the subsequent residues 172 – 327 forming the other lobe. The active

site cleft lies between the two lobes which are structurally similar and thought to have arisen after duplication of an ancestral gene.

Pepsin is composed of a high proportion of beta sheets and relatively few alpha helices.

Each lobe of the enzyme contributes one aspartic acid residue to the catalytic apparatus and in pepsin, the catalytic aspartic acid residues are at positions 32 and 215. It is the essential role of these residues to co-ordinate a water molecule for nucleophilic attack.

Pepsin has a broad range of substrates and also demonstrates esterase activity.

1.2.3.2.2 Papain (Liener, 1974)

Papain is a cysteine endopeptidase (MW 23,000Da: Isoelectric point pH 9.6) and a major protein constituent of latex from the melon-like green fruit of the small softwood tree *Carica papaya*. Since native crystalline papain is unreactive until acted upon by mild reducing agents, such as cysteine, it may exist as a zymogen. A single peptide chain of 211 residues is folded into two parts that form a cleft. The enzyme has a broad range of substrates and also demonstrates esterase activity.

Pepsin and papain cleavage points of human IgG 1 are shown in Figure 1.2a.

1.2.3.2.3 Trypsin (Walsh, 1970)

Trypsin is a serine endopeptidase (MW.23,800: Isoelectric point pH 10.5) produced as an inactive precursor called trypsinogen in the pancreas. It is cleaved initially by a specific enteropeptidase secreted in the duodenum to produce a small amount of active trypsin which can then activate more trypsinogen. Preferentially cleaves substrates containing Arg or Lys.

1.2.3.3 Genetics (Branden and Tooze, 1991; Lesk and Tramontano, 1992)

The genetic information for antibody diversity is contained in about 1000 small segments of DNA. The gene segments encoding the variable and constant regions of antibodies are clustered in three gene pools on separate chromosomes. One is for the heavy chain and one each for the two light-chain isotypes. In the heavy-chain gene pool, the variable domain is encoded by three types of segments, V, D, and J. The V segment codes for about the first 90

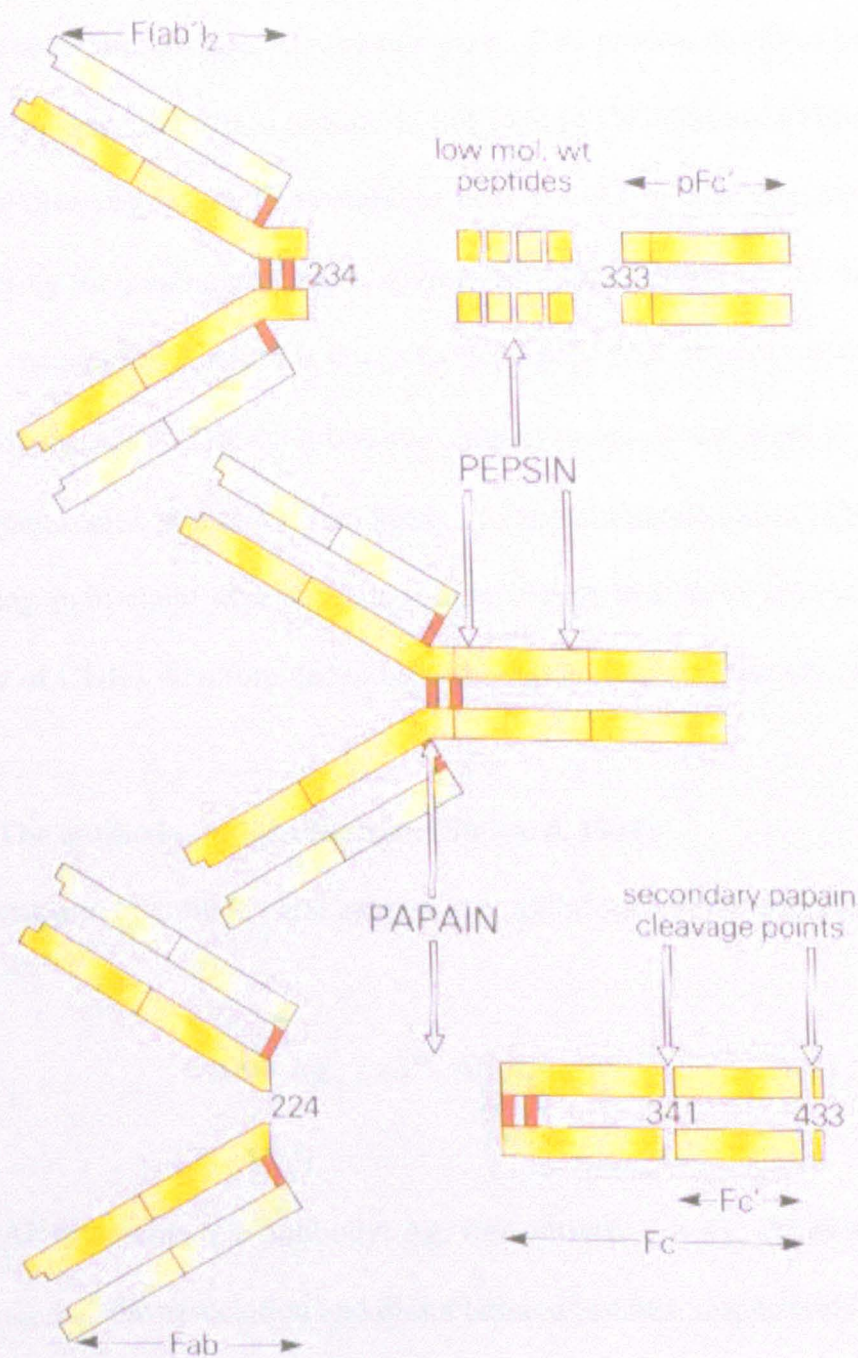


Figure 1.2a Cleavage of human IgG1 by pepsin and papain. Numbers refer to the Amino acid sequence. Trypsin primarily cleaves the molecule at position 222. Adapted from Steward (1981)

residues, D for the hypervariable region CDR3, and J for the remaining 15 residues of the variable domain. There are about 1000 different V segments, about 10 different D segments of variable lengths and about 4 different J segments in the heavy-chain gene pool. The DNA for the variable domain of a new B cell is assembled by random joining of one of each of these segments into a single continuous exon. This process is called combinatorial joining. Since the joining of these segments is not precise this creates additional diversity, called junctional diversity, where D recombines with V and J. In addition, extra nucleotides can be added during the joining procedure, giving still further diversity. As discussed above, the D segment encodes CDR3, which is thus a focus of particular diversity in the heavy chain.

The newly created V-D-J exon becomes joined to one of the eight C segments that encode the constant region domains of the heavy chains as separate exons. The genetic mechanisms underlying light-chain diversity are similar except that there are no D segments, and the diversity of CDR3 therefore depends largely upon junctional diversity generated during V-J joining.

1.2.3.4 The antibody-antigen reaction (Steward, 1981)

The interaction of antibody and antigen at equilibrium may be expressed in the following way:



where Ab represents free antibody; Ag, free antigen; Ab-Ag, the antibody-antigen complex; and k_a and k_d , the association and dissociation constants, respectively.

Applying the law of mass action.

$$k_a[\text{Ab}][\text{Ag}] = k_d[\text{Ab-Ag}] \quad (2)$$

Thus the equilibrium constant or affinity (K) may be calculated:

$$k_a/k_d = K = [\text{Ab-Ag}] / [\text{Ab}][\text{Ag}]$$

The intermolecular forces which contribute to the stabilization of the complex are the same as those involved in the stabilisation of the specific configuration of proteins and other macromolecules. Antibody affinity may be considered as the summation of attractive and repulsive non-covalent intermolecular forces resulting from the interaction of the antibody binding site and the antigen.

1.2.4 Applications of immunoglobulins

1.2.4.1 Diagnostic

1.2.4.1.1 Classification of immunoassay procedures

All immunoassays depend on the reversible, non-covalent binding of the analyte by the binding sites of specific antibodies in a reaction that obeys the Law of Mass Action.

They can be categorised into three groups. The first require no labelled reactants, and are based on the detection of large antigen-antibody complexes, either as a precipitation line in a gel or by turbidometric or nephelometric means. These are relatively insensitive techniques relying on the formation of optically detectable lattices of antibody/antigen molecules. The second involves use of a labelled antigen to act as a 'tracer'(Edwards,1985). The possibilities of using a labelled antigen, which allows the differential distribution of antigen between antibody-bound and free phases to be monitored, was first explored in 1960 by Yalow and Berson and by Ekins for the quantitation of human insulin and human thyroxine respectively. Separation of the phases is not always essential. Thus if the characteristics of the tracer in the bound and free phases are sufficiently different, then no separation is necessary. With its greatly enhanced detectability, the use of a radioactive tracer was a milestone in immunoassay technology and, in conjunction with improvements in radiolabelling techniques, revolutionized many fields such as endocrinology. Finally, there are immunoassays based on the use of labelled specific antibodies, which offer significant advantages in some circumstances.

There has been a steady move from immunoassays based on radiolabelled tracers to those based on enzymes, fluorescent or chemiluminescent molecules amongst others. Non-radioisotopic labels have gained increased popularity due to their long shelf-lives and to the avoidance of the potential risks associated with the handling and disposal of radioactivity.

On the debit side, most non-radioisotopic labels are susceptible to non-specific background interference from biological samples, resulting in the reduction of assay sensitivity. An important advantage of radioactive isotopes is that no endogenous material in a biological sample will interfere with their measurement (Edwards,1985).

1.2.4.1.2 Principles of fluorescence (Burrin, 1986)

Fluorescence is the phenomenon whereby a molecule, after absorbing radiation, emits radiation of a longer wavelength. This increase in wavelength is known as the Stokes' shift.

At room temperature most organic molecules are in the ground state and absorption of photons elevates electrons in these molecules to a higher energy state in less than 10^{-15} seconds. Energy is then lost very rapidly by collisional degradation (as heat), resulting in the energy of the excited molecules falling rapidly to that of minimal vibrational energy in the lowest excited state. The energy emitted from a limited number of molecules in regaining the ground state results in fluorescence.

The choice of a fluorophore is critical in designing a fluoroimmunoassay (FIA) and fluorescein (Figure 1.3) is the most widely used because it has a high quantum yield and absorbance spectrum in a range that allows excitation by a variety of light sources. Its emission maximum at ~522nm is suitable for most photomultiplier tubes, it shows no significant photolability under normal conditions, ambient temperature changes cause little effect on its fluorescence and many fluorescein-labelled materials have a virtually indefinite

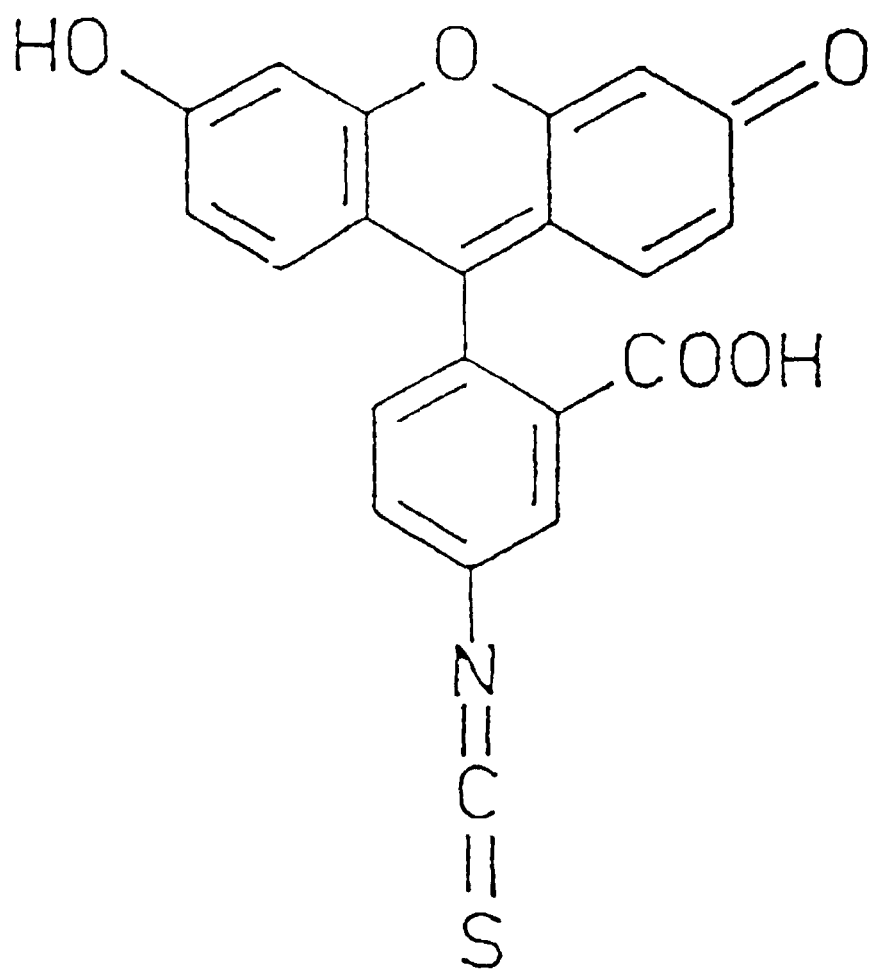


Figure 1.3 Structure of fluorescein isothiocyanate

shelf life. Its fluorescence life time of about 4 - 5 nanoseconds is appropriate for polarization measurement and, finally, it is hydrophilic and this may increase the solubility of the labelled compounds.

1.2.4.1.3 Fluorimetry (Burrin, 1986)

In a typical fluorimeter (Figure 1.4) a solution of fluorescent molecules is excited with light of an appropriate wavelength. This light (usually from a xenon or mercury lamp) is focused by an optical system and the desired wavelength isolated by a monochromator or filter before passing through into the sample compartment. A second optical system at right angles to the first directs emitted light via another filter or monochromator onto a photomultiplier tube, which is connected to an amplifier and a signal recording or display system.

When the fluorescent molecules in solution are excited at a suitable wavelength, the total intensity of emitted fluorescence is proportional to the intensity of exciting light, the concentration of the fluorophore, its extinction coefficient and its quantum yield.

1.2.4.1.4 Types of fluoroimmunoassay (Chard, 1990)

FIA methods may be classified into assays that require a separation step and those that do not. Only separation FIA are relevant to the experimental work described later. Thus the separation step has two important functions:

- 1) To separate the bound and free fractions of the fluorescein-labelled antigen.
- 2) To allow the removal of non-specific fluorophores or other interfering substances present in the sample prior to signal measurement.

The second function enables a greater sensitivity than can be obtained by non-separation FIA. In order to increase sensitivity a washing step can also be introduced. Precision can be maximized by introducing solid phase techniques such as covalently coupling the antibody to magnetizable particles, activated glass or Sepharose as described later.

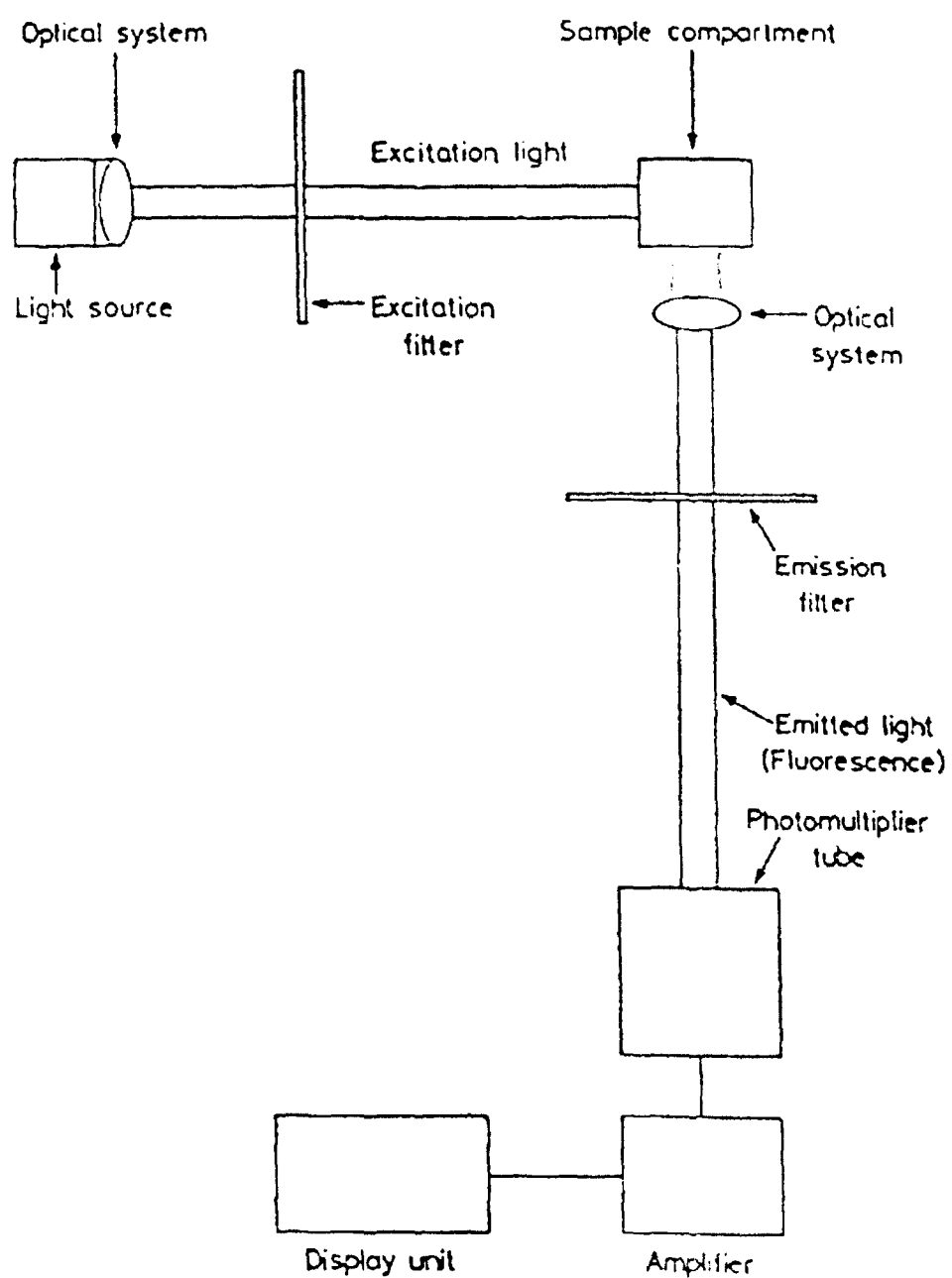


Figure 1.4 Schematic of the components of a simple fluorimeter

1.2.4.2 Therapeutic (Landon and Chard, 1995)

There is virtually no limit to the potential of antibody-based products for therapeutic purposes and there has been a general (probably misconceived) acceptance that monoclonal antibodies will dominate.

At present, monoclonal antibodies have not enjoyed the same success when used therapeutically as they have for diagnosis. Many believe that such lack of success is only temporary and will be corrected by the replacement of murine monoclonal antibodies by their humanised or human equivalents. Others consider that the choice between a monoclonal and a polyclonal antibody for a particular clinical situation will depend in large part on the mechanisms involved.

Some therapeutic applications of exogenous antibodies are listed in Table 1.2.

1.2.4.2.1 Snake antivenoms

We have identified six activities involved in the provision of antivenom (Figure 1.5) and their general characteristics are described below:

1.2.4.2.1.1 Venom supply

This involves the collection of large numbers of snakes from several sites within the region where the resultant antivenom will be used. The snakes are usually housed in a herpetarium and their venom collected regularly, usually monthly.

Venom is a complex mixture of substances, mainly proteins, produced by a specialized seromucous gland and inoculated under pressure by modified teeth (fangs) into the tissues. Its variability has been reviewed extensively by Chippaux et al. (1991) and is due to both different concentrations and biochemical differences between certain venom components (Nkinin et al. 1997). Thus it is essential to ensure that pools of venom have been sourced from the appropriate species of snake and retain their main components in active form. The toxicity of the pool is measured by *in vitro* assays to determine the dose which kills 50% of

For oral use

- To prevent and/or treat gastrointestinal infections
- To prevent absorption of drugs and other potentially toxic compounds

For systemic use as therapy

For replacement in immunodeficiency states

To bind to and thereby neutralize the biologic effects of :

- Haptens Cardiac glycosides, steroid hormones, tricyclic antidepressants
- Macromolecules Toxic constituents of snake, scorpion and spider venoms
- Microbial endotoxins and exotoxins
- Mediators of toxic shock
- Platelet-derived and growth factors
- Human chorionic gonadotrophin for fertility control
- Autoantibodies in idiopathic thrombocytopenic purpura

To bind to and thereby block cell surface epitopes:

- Receptors Such as that for IL-1 or the Fc part of immunoglobulins
- Adhesin molecules
- Glycoproteins Such as those necessary for platelet aggregation

To bind to cell surface epitopes and thereby help to destroy the cell:

- Examples include CD3 on T lymphocytes
- Rh(D) on neonatal red cells
- Tumour associated antigens on haematologically derived and solid tumours
- IgE-bearing cells
- Microorganisms including parasites, bacteria and viruses

Catalytic antibodies

For systemic use to deliver therapy

- Antibodies attached covalently or non-covalently to:
- Radionuclides(yttrium-90)
- Foreign toxins(ricin A chain, diphtheria toxin)
- Enzymes(to convert prodrugs to drugs or to bring tPA or urokinase to the site of thrombus)
- Drugs(adriamycin)
- Natural protective molecules(TNF, IL-2)
- Cytotoxic cells, using bifunctional antibodies

For extracorporeal use

- With blood (to remove apo B)
- With bone marrow(to remove T lymphocytes or neoplastic cells)

Table 1.2 Some therapeutic applications of exogenous antibodies (Landon and Chard, 1995)

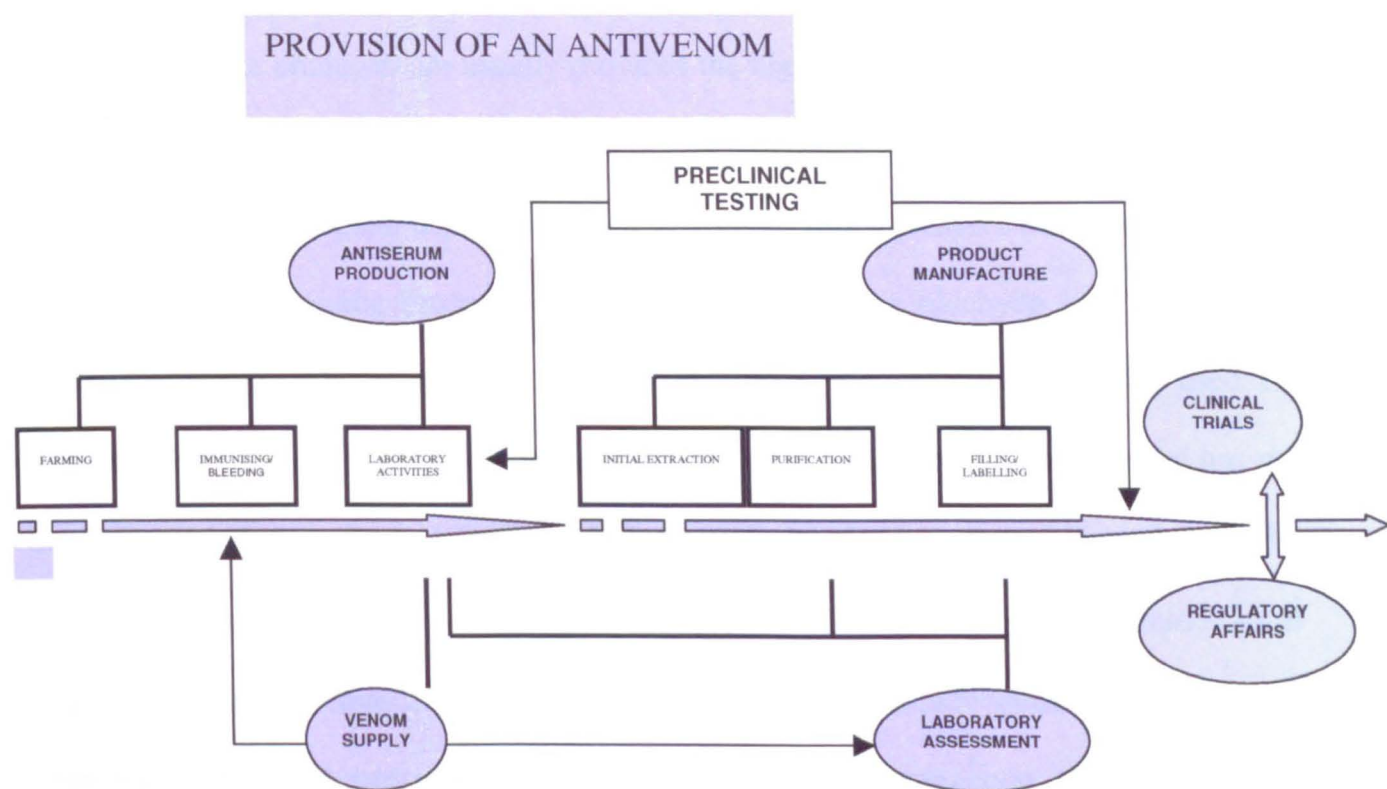


Figure 1.5 Six activities involved in provision of antivenom

a group of animals (LD₅₀). The most common animal used is the white mouse.

1.2.4.2.1.2 Antiserum production

Inoculation of the crude venom usually provides the highest titre; however, whole venom is often badly tolerated by the animal (Chippaux and Goyffon, 1998). As a result toxoids have been prepared by biological detoxification of the venom which preserve its immunogenicity. The most usual toxoiding procedures are complexing with an aldehyde such as formalin (Ramon, 1924) or glutaraldehyde. Detoxified or not, the venom preparation used for immunisation is often associated with an adjuvant. The precise role of the adjuvant has not been elucidated (Bomford, 1989) but it is thought to act in a controlling manner by decreasing the rate of release of venom and thus stimulating further the immunological response. The most commonly used adjuvants are Freund's (Chotwiwatthanakun et al. 2001; Sriprapat et al. 2003), bentonite (Sunthornandh and Ratanabanangkoon, 1994), aluminium hydroxide (Rucavado et al. 1996) and sodium alginate (Angulo et al. 1997).

The immunisation protocols depend on the toxicity and the immunogenicity of the venom, the animal model used for immunisation and the quality of the immune response of the animal (Chippaux and Goyffon, 1991). The optimum dose for immunisation is generally obtained by trial and error to obtain a sufficient antibody titre. Ten to fifty injections performed over a period of 3 to 15 months may be necessary to obtain hyperimmunisation (Chatterjee et al. 1968). The preferred animal for immunisation is the horse because of the large blood volume available, but other species can also be used (Russell et al. 1970; Carroll et al. 1992; Sjöström et al. 1994).

Antivenom is monospecific if only one venom is used, or polyspecific if the animal being immunised receives a mixture of venoms from different species.

1.2.4.2.1.3 Product manufacture

Although crude serum was originally used for therapy, for many years antivenom has been purified by successive steps in order to reduce anaphylactic reactions (Christensen, 1966; Kirkpatrick, 1991; Grandgeorge et al. 1996).

After elimination of the cellular elements by centrifugation, non-immune proteins and especially albumin, are discarded by precipitation with ammonium or sodium sulphate. The immunoglobulins are digested using either pepsin to produce $F(ab')_2$ fragments (Sanchez et al. 2003) or papain to produce a smaller Fab (Meyer et al. 1997; Ariaratnam et al. 1999) fragment. These purified products are then ampouled for marketing, either in liquid form or more rarely as a lyophilised preparation. Conventionally the times of expiry are usually 3 and 5 years respectively.

Before final packaging the antivenom is submitted to various controls: bacteriological culture in an appropriate medium, toxicological studies using animal inoculation for pyrogenicity and immunological studies to measure the neutralising efficiency of the product.

1.2.4.2.1.4 Laboratory assessment

Any unit raising antisera and intending to manufacture antivenoms needs to provide a range of microbiological, biological and analytical procedures (Table 1.3). Thus it is essential to ensure that pools of venom have been sourced from the appropriate species of snake and retain their main components in active form; that all animals being immunised are mounting an adequate humoral immune response ; and in particular that the vials of antivenom that have been manufactured are likely to be both effective and safe.

1.2.4.2.1.5 Clinical trials and regulatory scrutiny

At a recent WHO meeting it was noted that most antivenom centres “accepted the standard maximum lethality assay (LD_{50} of venom and ED_{50} of antivenom) as the yardstick for assessing antivenom preparations”. However, it was appreciated that the results didn’t always correlate with clinical efficacy (Theakston et al. 2003). Indeed the efficacy and

Microbiological	
Of manufacturing facilities	- regular viable and non-viable particulate count on air sample
Of manufacturing staff	- multiple settle plates and swabs
Of final product	- regular finger "dips"
	- bioburden
	- LAL
	- sterility using PH Eur membrane-filtration method
Biological	
Of venom	-LD ₅₀
Of final product	-ED ₅₀
	- pyrogen testing in rabbits
	- safety testing in mice and guinea pigs
General	
Of venom	- HPLC to ensure correct source
Of water-in-oil emulsion	- drop and standing tests to ensure stability
Of individual antisera	- ELISA or small scale affinity chromatography at week 14 on each individual sheep
Of antivenom pools	- gel filtration chromatography
	- protein concentration
	- Fc and albumin
Of final product	- ampoule integrity
	- colour/clarity/particulates
	-pH
	- protein concentration
	- Fc and albumin
	- gel filtration chromatography
	-ED ₅₀

Table 1.3 Summary of laboratory assessment techniques

freedom from side-effects of a new antivenom can only be assessed by means of a suitable clinical trial and the WHO meeting concluded “ This neglected field (clinical trials) should be given a much higher priority in the future”.

The results of clinical trials play an important role in deciding whether, or not, the appropriate regulatory authorities will allow a new product to be used routinely. These authorities will also want assurance that there has been some virological screening of the animals being used to raise antisera (such as in the case of horses, screening for equine infectious anaemia and African horse sickness). The regulatory authorities also insist on specific data for the production process for each specific product and ban the use of anti-microbial agents such as cresol and toluene. Hence, the need for a robust, simple, platform technology and a clean manufacturing environment.

1.2.5 Side-Effects (Sutherland, 1977; Landon and Chard, 1995)

The benefits of therapy must outweigh any risk involved and every effort must be made to reduce the incidence and severity of side effects to a minimum. This can be achieved only if all deleterious effects are recognized and if mechanisms are fully understood. The risks of passive immunization are considered under three headings in Table 1.4. Those that are an inevitable consequence of specific binding by the therapeutic product; those of an allergic nature; and finally a range of non-specific effects.

Allergic responses are described in more detail below because this is the main area of concern with passive immunization. Hypersensitivity reactions range from a mild rash to severe, sometimes fatal anaphylactic shock; all are examples of an inappropriate immune response that may lead to tissue damage. In general, the less pure the protein, the more foreign its nature and the larger the amount given, the greater is the risk of an allergic reaction.

Type I anaphylactic hypersensitivity requires prior exposure to the foreign antibody (or contaminating protein) and it is mediated by IgE. Specific IgE secretion is stimulated by the

first exposure. Some becomes attached to Fc receptors on mast cells. A second exposure to even small amounts of the protein leads to bridging between two IgE, with degranulation of mast cells and release of histamine and other vasoactive molecules. Clinical manifestations occur within minutes of re-exposure and can include tachycardia, hypotension, bronchospasm and angioneurotic oedema. Type I hypersensitivity reactions were common with equine-based products due, for example, to prior exposure to tetanus antitoxin derived from horses.

Type III immune-complex-mediated hypersensitivity is more common than type I reactions and is mediated by IgG. Each IgG molecule or its $F(ab')_2$ has two binding sites which permit “cross-linking” to occur if the antigen has more than one epitope. Thus immune complexes can be formed comprising many antibody and antigen molecules. Some immune complexes remain in solution in the circulation; others precipitate out or become fixed to the wall of a blood vessel. All immune complexes can cause platelets to aggregate and release vasoactive amines such as serotonin. Macrophages attach to the Fc and initiate phagocytosis and release cytokines. The simplest example of mechanisms by which immune complexes are formed is when exogenous antibodies bind macromolecules (such as the constituents of a snake venom) already present in the patient. Much more common are primary (delayed) and accelerated serum sickness, in which the patient’s own antibodies are involved.

1.3 Transmission of avian immunity to the egg

1.3.1 History and general introduction

In 1893, Klemperer showed that antibodies produced in the hen as a result of an immunogenic challenge transfer from the circulation to the egg yolk. Fraser and his colleagues (1933) conducted two studies, the first in hens and the second in ducks. These showed that immunisation of the birds with diphtheria toxoid resulted in the appearance of antitoxin both in their serum and in the yolks of their eggs. The serum antitoxin concentration fell rapidly after injections were discontinued at a time when the birds began

1. Due to specific effects of the exogenous antibodies

Recurrence of cardiac problems, with excess anti digoxin Fab
Characteristic symptom complex following first administration of antilymphocyte globulin and OKT3 due to T lymphocyte destruction
Increased incidence of lymphoproliferative disorders following use of OKT3
Potential deleterious effects due to blocking biologically important molecules

2. Allergic response

(i)To exogenous antibodies(or contaminating proteins)

- Type I anaphylactic hypersensitivity
- Type III immune-complex-mediated hypersensitivity
 - primary(delayed) serum sickness
 - secondary(accelerated) serum sickness

Humoral immune response blocking or enhancing clearance of exogenous antibodies
Possible immunomodulation

(ii)By exogenous antibodies

Type III immune-complex-mediated hypersensitivity
-involving foreign antibodies as the binding reagent with infusion of antibody aggregates, including idiotype:anti-idiotype complexes

3. Non-specific side-effects

Microbial contaminants

- endotoxins
- bacteria
- mycoplasma and fungi
- conventional human, rodent and bovine viruses
- slow viruses

Other contaminants such as prions
Potential tumorigenicity
Cardiac
Renal

Table 1.4 Some potential side-effects associated with the systemic administration of exogenous antibodies (Landon and Chard, 1995)

to lay eggs and the concentrations of antitoxin in the yolk were found to correlate directly with the concentrations in serum. In 1946, Brandley et al used hens which had been immunized with, or recently recovered from Newcastle virus to show transfer of specific antibodies to the egg yolk.

1.3.2 Antibody production from chicken eggs

The inherent advantages of chicken eggs for antibody production are listed below based on conclusions drawn from a series of five papers (Kuhlmann et al. 1988; Schmidt et al. 1989; Wiedemann et.al. 1990; Jungling et al. 1991; Wiedemann et al. 1991).

These are:

1. The productivity of eggs is high and one egg contains approximately 15ml yolk with at least 8g/l of chicken antibody.
2. Collecting eggs is easier than taking blood and less stressful for hens.
3. Eggs can be stored at room temperature for several weeks.
4. It may be better to immunise several individual hens with single different microbes rather than each individual with multiple different microbes. If necessary a "cocktail" can then be obtained by mixing individual eggs.
5. Chickens, via egg yolk, can provide small amounts of antibody of nearly constant quality over a long time while colostrum is only available for 1 to 2 days.
6. Chickens are more effective antibody producers, being able to produce approximately 20 times more antibody quantity per kg bodyweight than does the cow in the colostrum. Also it is less expensive to house 20 chickens than 1 cow.

1.3.3 Chemical and physical aspects of a typical egg

The simplistic classification of the egg into three distinct fractions, the yolk (ovum), white (albumen) and shell (calcified exterior, cuticle and membranes) is used in Tables 1.5, 1.6 and 1.7 to create a physical and chemical picture of a typical chicken's egg (Gilbert, 1967).

I have included a schematic of a typical hen's egg (Figure 1.6) to illustrate why it is simplistic to divide the egg into three fractions but the sub-categories of these three fractions will not be discussed.

1.3.4 Reproductive anatomy of the chicken (Gilbert, 1967)

The following is a brief description of how and where in the chicken, the main events in the formation of yolk, white, and egg shell occur. In the hen only the left ovary is functional since its early oestrogenic secretion prevents the right from developing. Removal of the left ovary results in the right ovary developing into a testis. When the hen becomes sexually active at about 20 weeks, there is a hundred-fold increase in ovarian weight due to developing follicles. In the sexually active bird the oviduct is a tube some 80cm long extending from the single ovary to the cloaca and occupying a large part of the abdominal cavity. The development of the oocyte into a yolk takes place entirely in the ovary and can be divided into three phases. First a period of slow growth lasting for months or even years, following which the oocyte measures only 1 mm. The second phase lasts about 60 days after which the oocyte measures about 6mm. During this phase large vacuoles appear and this type of yolk is called white yolk and has a relatively high protein content. Finally there is a rapid growth phase during which the main mass of yellow yolk is deposited and the yolk reaches its full size (19.0g). This takes about 7-11 days and terminates just prior to ovulation.

The oviduct can be divided into five parts; infundibulum, magnum (albumen secreting part), isthmus, uterus (shell gland) and vagina. During ovulation the oviduct synthesises the coats which surround the ovum, the magnum forming the albumen, the isthmus the shell membranes and the uterus the shell, pigment and cuticle.

The vagina does not contribute to the formation of the egg. Figure 1.7 illustrates a schematic of the five-part structure of the oviduct.

1.3.5 Immunoglobulin classes in the chicken

Leslie and Clem (1969) concluded that the domestic hen has at least two immunoglobulin classes. One, with a molecular weight of approximately 900,000 Da, is very similar to mammalian IgM. The other has a similar structure to mammalian IgG but with sufficient differences for the author to give it a different name, IgY. The differences between avian IgY and mammalian IgG are listed in Figure 1.7a and Table 1.8 (Landon and Chard, 1995).

Weight	58g
Long axis	5.7cm
Short axis	4.2cm
Long circumference	15.7cm
Short circumference	13.5cm
Volume	53.0cm ³
Gross surface area	68.0cm ³

Table 1.5 General characteristics of the egg.

	Percentage of whole	Water; Percentage of total egg	Solids; Percentage of total egg
White	58.0	76.2	20.0
Yolk	32.0	23.6	51.0
Shell	10.0	0.2	29.0

Table 1.6 Relative composition of the egg.

Chemical Class	Shell	White	Yolk
Water	1.0	88.5	47.5
Protein	4.0	10.5	17.4
Lipid	N.A.	N.A.	33.0
Carbohydrate	Some in protein	0.5	0.2
Inorganic ions	95.0	0.5	1.1

Table 1.7 Percentage composition of the shell, white and yolk (Gilbert, 1967).

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Rose et al (1974) concluded that immunoglobulin classes in the hen's egg are segregated between the yolk and white. Thus the egg yolk contains at least two antigen-binding subclasses of IgY derived from the hen's serum but no IgM or IgA classes while the egg white contains IgM and IgA but no IgY.

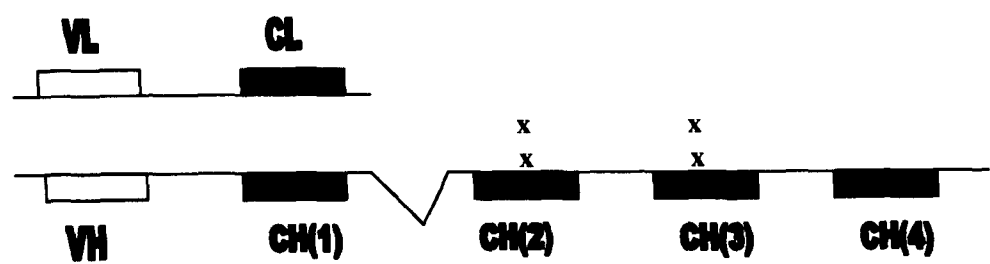
1.3.6 Composition of egg white and egg yolk

The composition of egg white is summarised in Table 1.9 (Gilbert,1967).

The micro structure of egg yolk was described by Bellairs (1961). It consists of large spherical bodies (25-150µm diameters), distributed in a continuous phase, and smaller granules (up to 20µm diameter) found both within the yolk spheres and in the continuous phase. Egg yolk contains approximately 50% water and the remainder is predominantly proteins and lipids in the proportions of 1:2; the lipid is present as lipoprotein. The proteins and lipoproteins are partitioned between the yolk structures (Cook 1968). The granules, which may be obtained by centrifugation of diluted yolk, account for approximately 23% of the yolk solids. They contain a phosphoprotein termed phosvitin, and two high density lipoproteins, the lipovitellins, in a complex in which most of the yolk calcium and iron are present (Burley and Cook, 1961). They also contain a small amount of low density lipoprotein. Phosvitin and the lipovitellins account for about 4% and 16% of yolk solids respectively.

During centrifugation the yolk spheres disintegrate, the granules they contain sediment, and the supernatant is thus derived partly from the spheres and partly from the continuous phase. The supernatant contains a low density fraction which can be obtained by flotation in an ultracentrifuge and which consists of low density lipoprotein containing only about 12% protein. It accounts for 65% of yolk solids and 95% of yolk lipid. The supernatant also contains a heterogeneous group of water soluble proteins, the livetins, which together account for less than 10% of the yolk solids.

Chicken IgY



Mammalian IgG

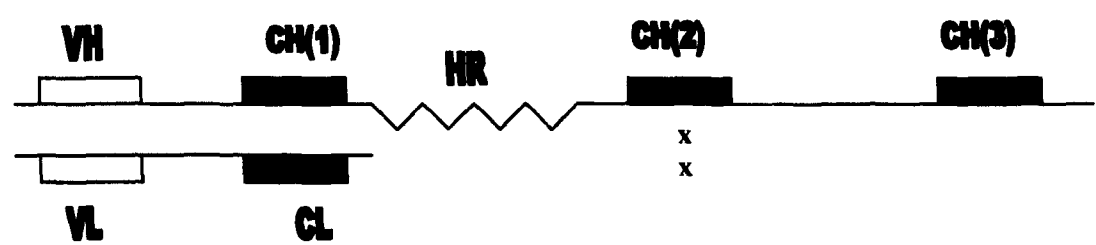


Figure 1.7a
A simplified model of a mammalian immunoglobulin compared with an avian immunoglobulin. Differences are revealed in the number of constant domains of the heavy chain, the carbohydrate chain(x) and the hinge region(HR) which is much more evident in the case of IgG. L = light chain, H= heavy chain, V= variable domain, C=constant domain. Adapted from (Schade et al. 2001).

	Mammalian IgG	Hen IgY
Structure	Comprise 2 heavy and 2 light chains	Comprise 2 heavy and 2 light chains
Molecular weight	About 160,000	About 180,000
Hexose chain	1%	2.22%
Mean pI	7.8	6.8
Pepsin Digestion	To F(ab') ₂ + Fc	To x2 Fab +Fc
Papain Digestion	All to x2 Fab + Fc	Only about 70% to x2 Fab + Fc
In 1.15M Saline	No aggregation	Considerable aggregation
Binding to:		
Complement	Yes	No
Macrophages	Yes	No
Protein A	Yes	No
Protein G	Yes	No
Rheumatoid Factor	Yes	No

Table 1.8 Comparison of mammalian IgG and avian IgY. Adapted from (Landon and Chard, 1995)

Egg white solids	Percentage of total solids
Ovalbumin	54.0
Ovotransferrin	13.0
Ovomucoid	11.0
Ovomucin	1.5-2.9
Lysozyme	3.5
G2-Globulin	4.0?
G3-Globulin	4.0?
Ovomacroglobulin	0.5
Ovoglycoprotein	0.5-1.0
Flavoprotein	0.8
Ovoinhibitor	0.1 -1.5
Avidin	0.05
Unidentified proteins	About 8.0

Table 1.9 Representative composition of egg white solids (Gilbert, 1967)

Patterson et al. (1962) demonstrated the transfer of [¹³¹I]- γ globulins from the blood to the developing ova. Martin and Cook (1958) isolated γ livetin and Williams (1962) provided immunoelectrophoretic evidence that it was largely plasma γ -globulin.

1.4 Venomous Snakes

1.4.1 General introduction (Mattison, 1995)

It is accepted generally that snakes evolved from their closest relatives the lizards and that they first appeared between 100 and 150 million years ago. Between then and now they have evolved into approximately 2,400 currently recognized species.

Colubrid snakes appeared approximately 60 million years ago. Approximately two thirds of all snakes belong to the family Colubridae and are found throughout the temperate, tropical and desert regions of the world. These generally have either rear fangs or solid teeth and their small fangs usually render them harmless to man, although the Montpellier snake (*Malpolon monspessulanus*) of the Mediterranean region, the cat snake (*Telescopus fallax*) found in south-eastern Europe, and the African boomslang (*Dispholidus typus*) can inflict dangerous bites. The venom of Colubrids is usually of low toxicity to man, and the small volumes that can be obtained from individual snakes has resulted in the chemistry of the venoms from this family being relatively poorly studied.

Approximately 25 million years ago representatives of two other important families of venomous snakes, the Elapidae and the Viperidae appeared.

The Elapidae is the second largest family and contains nearly half of all known venomous snakes. They are distributed throughout Africa, Asia, the southern parts of North America, Central and South America and Australasia. The snake fauna of Australia is unique in that the elapids constitute the majority and also represent the widest variety of forms.

The Elapidae are thought to have evolved from the Colubridae, from which they differ in having a more efficient system for injecting venom; the fangs are effectively tubular and positioned at the front of the mouth where they may be brought into use more easily.

Most of the remaining venomous snakes are to be found in the major family, Viperidae. They are distributed all over the world with the exception of Australasia. The Viperidae is further divided into two sub-families (Figure 1.8), the largest sub-division being represented by the Crotalinae which are better known as pit vipers.

However, the number of species is no indication of abundance and the sub-family Viperinae contains some of the most widely distributed venomous snakes. Indeed, one of these, *Vipera berus*, has the widest known distribution of all the world's snakes. All the members of the Viperinae are restricted to the eastern hemisphere.

Atractaspidae is a small family of venomous, burrowing snakes restricted to Africa and the Middle East.

Antivenoms against venoms of snakes of the genus *Vipera* will be studied in this thesis.

1.4.2 European venomous snakes (Phelps, 1981)

The phylogenetics and systematics of the Vipers may be determined by: (1) external morphology (2) ethology (3) immunological studies and (4) comparison of chromosomal characteristics. Most of the European vipers are stout-bodied with a short tail and a distinctive flat and triangular head (Figure 1.9). They are slow moving, usually lie in wait for their prey and strike when it comes within distance. Their diet consists mainly of small rodents. Hibernation usually takes place from October to April and mating occurs following emergence in the spring with the young being born in the summer or fall. Most genera are ovo-viviparous, the young being born enveloped in a membrane from which they immediately free themselves. Some sub species of *V.lebetina* and *V.palestinae* lay eggs.

Most adapt readily to captivity, the notable exception being *V.berus*, which is very difficult to rear and maintain successfully.

1.4.2.1 Epidemiology

1.4.2.1.1 Snake species distribution

Snakes are poorly represented in Europe, for two reasons. Firstly the number of species is

generally higher in warmer than in temperate zones and secondly the geologically recent ice ages in Europe had a devastating effect on the survival of cold blooded animals.

The most common naturally occurring venomous snakes in Europe belong to the family Viperidae, genus *Vipera* (Figure 1.8). *V.berus*, *V.aspis* and *V.ammodytes* are the most widely distributed venomous snakes in Europe (Steward,1971). A few venomous colubrid species also occur in Europe, including *Malpolon monspessulanus*, which may be of medical importance.

V.berus occupies a larger land area than any other poisonous snake. It occurs in almost the whole of Europe below the arctic-circle except for Ireland, the more southerly parts of the continent and the larger Mediterranean islands. It extends eastwards through Asia to the Pacific Ocean (Steward,1971). *V.aspis* occurs in vast regions of France and Italy, in the Pyrenees and the Alps, but it has been reported from other parts of south Europe and southern Germany. *V.ammodytes* is widespread in south-eastern Europe and is also found in northern Italy, Austria and Turkey. *V.ursinii* occurs in restricted areas in central and eastern Europe. *V. latastei* is common on the Iberian Peninsula.

V.lebetina and *V.xanthina* are found in Cyprus, Turkey, West Asia and North Africa. *V. palestinae* occurs in the Middle East (Klemmer, 1968).

1.4.2.1.2 Incidence of bites

The incidence of venomous viper bites in Europe is difficult to assess. Statistics from poison centres do not seem to be complete, as many cases apparently are not reported (European Commission report, 1996). An investigation by Pozio (1988) in Italy shows that 2,329 patients were admitted to 292 hospitals during 1980-1984 and 286 of the cases were studied in detail. Bites by *V.aspis* dominated heavily. In a Swiss report recorded over 16 years (Stahel et al. 1985), 113 cases of *V. berus* and *V. aspis* bites were studied. In France the number of viper bites has been estimated at around 2,000 per year with a few fatalities (Chippaux and Goyffon, 1989) and Claud et al.(1989) found that 80 patients had been

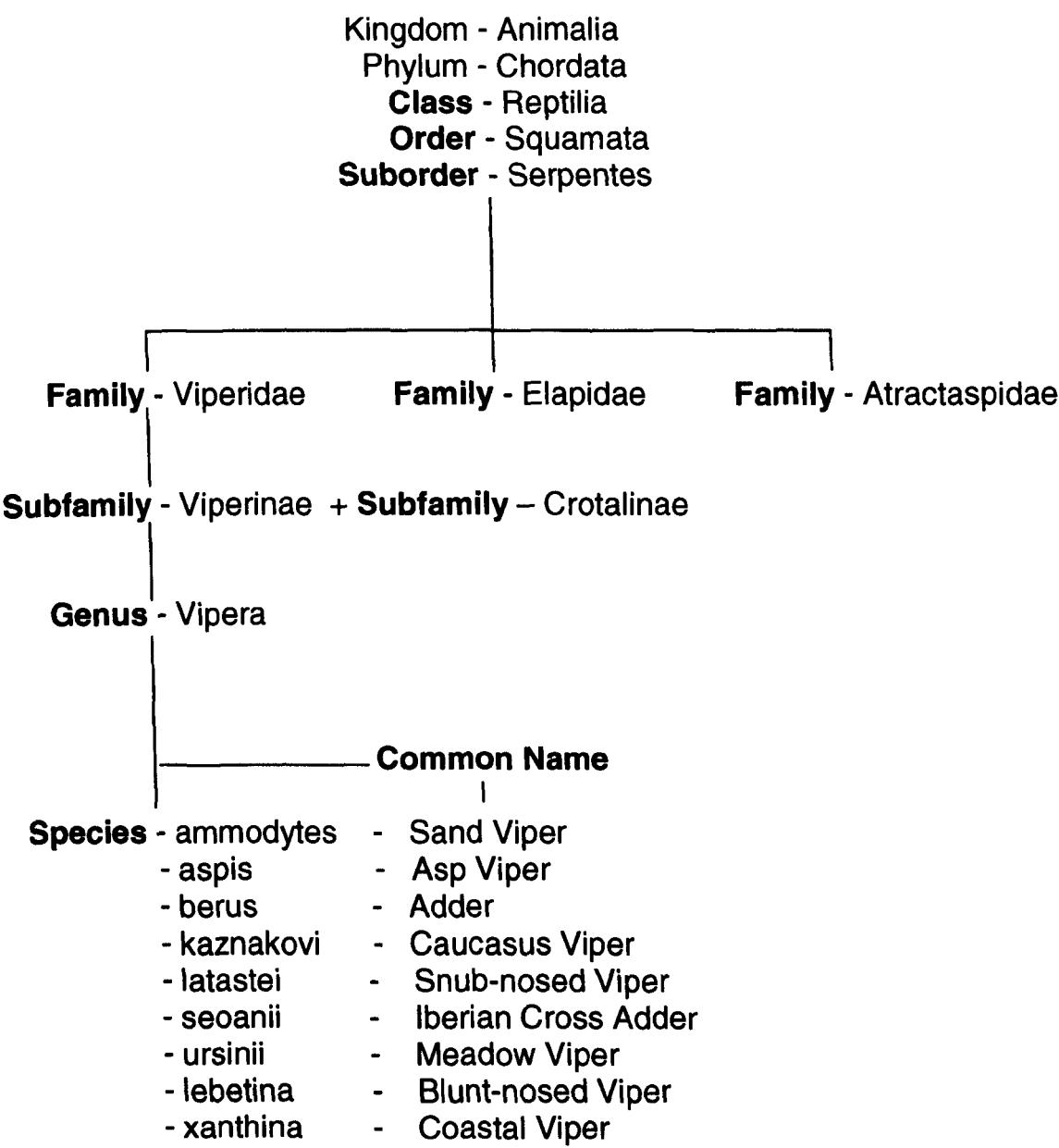


Figure 1.8 Classification of important venomous snakes.
Adapted from (Phelps, 1981; Mattison, 1995)

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Please refer to the original text to see this material.

treated for viper bite in one French hospital between 1980-87. In another French publication, seven serious envenomations are presented (period 1976–1986) in which three of the victims died (Rowsselot et al. 1991). In the United Kingdom the number of bites was estimated in the seventies to be lower, around 100 per year, with only one death since the last decade (Reid, 1976). In a study in Sweden (Persson and Irestedt, 1981) it was found that around 150-200 people are hospitalized annually because of *V. berus* bites. The total incidence of bites in Sweden was estimated in the 1950's to be around 1,300 per year (Marquard, 1951).

Estimations made by Gonzalez (1991) are of a total incidence of 15,000–20,000 venomous snakebites and 50 deaths annually in Europe as a whole. These figures may be accurate but difficult to assess.

Snake bite also presents a problem to domestic livestock, including cattle and horses.

1.4.2.2 Symptoms and signs

1.4.2.2.1 Toxins and mechanisms of action

The European vipers are closely related to each other and there are only minor differences in the composition of their venom and the symptoms of their envenoming. The venom contains a mixture of proteins with enzymatic and toxic activity such as proteolytic enzymes, peptide hydrolases, hyaluronidase, phospholipases A₂, phosphodiesterases and L-amino acid oxidase. In addition, there are amino acids, carbohydrates, toxic polypeptides and metallo proteinases (Persson, 1995).

A neurotoxin has been isolated from the venom of *V. ammodytes*, but it is questionable whether this has any clinical significance (Harris, 1982). There is also evidence of a neurotoxic component in *V. aspis* venom as certain neurological symptoms have been observed after bites (Scholer, 1970). The more dramatic central nervous system symptoms occasionally seen in envenoming by European vipers, like somnolence, unconsciousness,

urine and faecal incontinence and convulsions, have mostly been attributed to sudden and profound hypotension.

Local spread of the venom is facilitated by the action of hyaluronidase. Proteolytic enzymes cause damage to subcutaneous tissue structures including capillary and lymphatic endothelial cells with leakage of lymph, plasma and erythrocytes. This will, depending on the amount of venom injected, lead to extensive oedema that gradually undergoes haemorrhagic discolouration. Local and systemic haemolysis and coagulopathies may also be induced.

A spectrum of systemic effects may follow the release of highly active endogenous substances including histamine, bradykinin, prostaglandins and serotonin.

1.4.2.2.2 Circumstances. Route of exposure

Bites most commonly occur outdoors when people come across the snakes in their natural habitat. They occur mainly on the extremities (the hands or feet) in connection with picking wild berries and mushrooms or during gardening. Although much rarer, bites are also observed on the trunk, neck and head. They may happen during swimming or when lying down on the grass. *V.latastei*, may sometimes be found in trees, with a consequent increase in bites to the head or trunk, for example in farmers harvesting fruits.

The snake releases the victim directly after the bite. Bites can occur at any time of the day but not during the winter season in northern countries. Bites occur most commonly in warm and dry weather and the snakes are mostly found in sunny, rocky parts of forests, in meadow-land, in mountain districts (even at high altitude) and along the coasts. Vipers can both swim and climb.

Route of exposure is mostly intradermal or subcutaneous and rarely intramuscular. The venom cannot penetrate the skin.

1.4.2.2.3 Symptomatology

Most patients bitten by European vipers will have a relatively mild clinical course, but severe cases are constantly reported. The main features of envenoming are local tissue damage, gastrointestinal symptoms and systemic circulatory disturbances (Table 1.10). In addition, there is a wide variety of symptoms, many of which are related to the main features mentioned earlier.

Symptoms of systemic envenoming usually develop rapidly, often within minutes, but may also be delayed for many hours after the bite. The local reaction starts slowly and may continue to develop for two or three days. Many of the serious complications, like severe anemia, haemolysis, renal impairment, pulmonary oedema and bleeding are late complications.

1.4.2.2.4 Laboratory assessment

With viper bites the initial assessment should include pulse and respiration rate, blood pressure, temperature, evaluation of skin discolouration and the presence and extent of oedema. Haematological tests should include an erythrocyte count and the measurement of haemoglobin and haematocrit to indicate if there is any internal bleeding or haemolysis. The white cell count is a useful and simple non-specific test of systemic envenoming and values of $2 \times 10^{10}/L$ may indicate the need for antivenom. Most of the haematological changes involve coagulation and in severe cases, the prothrombin time and partial thromboplastin time are increased. The platelet count may be depressed although it is often normal. In severe cases changes in urine composition (protein, blood, albumin, glucose, urea and creatinine) should be monitored. Any cardiac abnormalities should also be checked for by an electrocardiogram and serum concentration of aspartate aminotransferase and/or creatinine phosphokinase. A low bicarbonate concentration reflects the metabolic acidosis that is a feature of severe experimental viper bite poisoning. Potassium concentrations are sometimes raised.

- Psychological reactions
- Local symptoms
- Gastrointestinal symptoms
- Circulatory disturbances
- Central and peripheral nervous system disturbances
- Renal dysfunction
- Respiratory symptoms
- Angioneurotic oedema
- Haematological changes
- Coagulopathy
- Others
- Late symptoms

Table 1.10 Envenoming by European vipers. Overview of symptoms (Persson, 1995)

1.4.3 *V. latastei latastei*

1.4.3.1 Description (Steward, 1971).

See Fig 1.9 (p. 42).

Full grown adults measure about 20 to 22in in length but exceptionally reach about 29in. The head is distinct from the neck. The snout is upturned and usually ends in a short, scaly horn, more or less obliquely tilted backwards. The ground colour is usually grey or brown, often reddish brown in females. Along the back is a series of black-edged, dark brown rhombs running together to form a wavy or zigzag stripe. A series of dark spots along the flank correspond with the indentation in the dorsal stripe. A dark streak runs from the eye to the angle of the mouth, and there may be small, dark markings on the top of the head. The undersides are yellowish or grey, speckled or marbled with black, and often with some white speckling. The underside of the tip of the tail is normally yellow, or spotted with yellow.

1.4.3.2 Species distribution

Lataste's Viper can be found in the western region of the Mediterranean. According to Saint Girons (1980), there are currently three sub-species. *V.l.latastei* is mainly found in the Iberian Peninsula, *V.l.gaditana* mainly in the southwest of the Iberian Peninsula and in North Africa and *V. l. monticola* in the Atlas mountains of Morocco, between 4,000 and 12,000ft above sea level. The latter is a dwarf subspecies 12in length.

1.4.3.3 Incidence of bites

According to Gonzalez the number of venomous snake bites in Spain was 1,500-2000 per year between 1980 and 1987 with between 2 and 8 fatalities annually during this period. *V.latastei* was responsible for approximately 80% of these cases and *V. aspis* and *V. seoanei* for the remainder. *V. latastei* is the most aggressive of the afore mentioned and its bite the most dangerous (Gonzalez, 1991).

Vipers are nocturnal reptiles but their habits change according to the climate, feeding and habitat. In Spain, *V.latastei* is more active during the day in spring and autumn. In

mountainous regions and during stormy summers they become diurnal, thus the incidence of bites increases. Also *V.latastei* may adopt arboreal habits, thus causing severe bites in the head, face and trunk (Gonzalez, 1991).

1.4.3.4 Venom toxicity

The venom of *V.latastei* has not been extensively studied. Salva Miguel (1946) reported that the venom possessed haemolytic, coagulant and anticoagulant actions. The latter could only be appreciated *in vivo*. Also experimentally, intravenous administration of the venom produced irreversible collapse of the peripheral nervous system.

Zeller (1948) compared the enzymatic activity of the venom with that of other Viperidae and found them to be similar. For a long time the venom of *V. latastei* was considered to be less dangerous than that of *V.aspis* as reported by Boulenger in 1913 in the first work in the English language dealing with snakes of Europe. It was later reaffirmed by Steward (1971), who may not have read the work by Salva (1946) who claimed the opposite in mouse studies. This contradictory evidence may be due to the fact that the toxicity of the venom of each species and sub-species is affected by many factors, including the time of year, climate and diet. Thus comparisons between venoms of *Vipera* species and subspecies may be difficult to make. Gonzalez (1991) claims that *V.latastei* bites result in a larger proportion of deaths than those of *V.aspis* and *V.seoani*. From studies of *in vitro* antigenic properties, Detrait et al.(1983) conclude that *V.latastei* venom is similar to *V.aspis* venom but very different from *V.ammodytes* venom. More importantly, Detrait et al. (1983) showed that *V.l.gaditana* venom is neutralized effectively by antivenoms against *V. berus*, *V. aspis* and *V.ammodytes*.

1.4.3.5 Clinical features (Gonzalez, 1991)

1.4.3.5.1 Local signs and symptoms

1.4.3.5.1.1 Fang marks

The distance between the fang marks varies from 5mm for young small -sized snakes to 20mm for adults. Subsequently, a small erythematous halo surrounds the marks, later becoming ecchymotic with a mild local haemorrhage.

1.4.3.5.1.2 Pain

Pain usually occurs immediately but its intensity is variable and may be mild or absent. If the patient has not seen the reptile, which is the case in approximately seventy per cent of cases, the bite may be mistaken for a puncture or a burn caused by a plant. The pain may disappear in minutes and sometimes indefinitely but can persist for a variable period.

1.4.3.5.1.3 Oedema

Oedema is the most reliable sign of local envenomation, because it occurs early and progresses during the first hours, when general features have not yet developed. Indeed envenomation is practically ruled out in its absence. It usually appears within minutes after the bite but, exceptionally, may be delayed up to 12 hours following general symptomatology. The oedema becomes highly painful on palpation and it extends within 36-72 hours. It may last for weeks and it may produce an inability to use the affected limb, for several months. An evaluation of the rapidity of development, extension and duration of the oedema will give an idea of the severity of the envenomation.

1.4.3.5.2 General signs and symptoms

General symptomatology varies in intensity. It may be absent in mild envenomations; in severe cases, however, it may appear immediately or within a few hours. Gastrointestinal and cardiovascular manifestations with extracellular dehydration are the most frequent features. Neurological, respiratory, renal and haematological (haemolysis and impairment of coagulation) manifestations are less frequent.

1.4.3.5.3 Complications

The need for amputation of the affected limb is infrequent in cases of *V.latastei* bite, although it is aggravated by the inadequate maintenance of tourniquets.

1.4.3.6 Treatment

Gonzalez (1991) has reported that out of 1,500-2000 bites per year in Spain between 1980 and 1987 (eighty percent of which are *V.latastei* bites) antivenom therapy was used in approximately fifty per cent of cases, with the remaining cases being successfully managed symptomatically. Antivenom was efficacious in restoring blood coagulation and resolving systemic symptoms. Gonzalez (1991) employed antivenoms from Pasteur and Lelong (France), Zagreb (Yugoslavia), Behringwerke (Germany), and Slavo (Italy). The best results were obtained with those from Zagreb and Behringwerke. Allergic reactions, including early anaphylactoid reactions and late (serum sickness) reactions, have been reported following administration of all antivenoms with the Behringwerke antivenom inducing mild reactions in 16 of his patients.

CHAPTER 2: IMMUNOLOGICAL METHODS FOR THE ASSESSMENT OF TOTAL AND SPECIFIC ANTIBODY CONCENTRATIONS

2.1 The development and application of a specific fluoroimmunoassay for chicken immunoglobulin Y

2.1.1 Background

Since Williams in 1962 established that chicken egg yolk was a useful source of antibodies, investigators have used these antibodies for both diagnostic (Polson et al. 1980; Schade et al. 1991) and therapeutic applications (Akita et al. 1992, 1993; Almeida et al. 1998). This has stimulated researchers to attempt to develop extraction and purification methods that are simple, rapid, easily scaled up and which preserve the activity of the IgY.

In the past investigators employed non-labelled immunoassays to monitor IgY recoveries (McLaren et al. 1994). However these methods are time consuming, require technical expertise to produce precise results and are dependent upon the molecular state of the antigen under study. More recently a one site (Losonczy et al. 1999) and a two site (Fischer and Hlinak, 1996) immunoenzymometric assay (ELISA) with a colorimetric end point were developed. However, such assays demand careful attention to factors such as temperature and timing.

We have developed a solid-phase fluoroimmunoassay for the quantification of IgY to compare the various extraction/purification methods with respect to IgY recovery.

2.1.2 Materials

Fluorescein isothiocyanate (FITC) isomer I and chicken immunoglobulin (IgY) were obtained from Sigma; ovine anti-chicken IgY serum (Batches 1013 and 1014) and an ovine anti-snake venom serum to act as a control (NSB) from MicroPharm Ltd; cyanogen bromide activated (CNBR) 4 Fast Flow Sepharose and Sephacryl S-100 from Amersham Pharmacia Biotech; Sartoclon Micro with a 30kDa pore size membrane from Sartorius;

ChromPure ovine and equine IgG from Jackson ImmunoResearch Laboratories, Inc; and chicken eggs (medium/class A) from Lion. All other chemicals were from Sigma.

2.1.3 Methods

2.1.3.1 Preparation of fluorescein-labelled chicken IgY

Chicken IgY (Molecular Weight = 180 KDa) and FITC (Molecular Weight = 389 Da) were reacted in a bicarbonate buffer (Pourfarzaneh et al.1981) overnight at a molar ratio of 1:3 by adding 1mL of IgY (16g/L) to 1mL of FITC (0.12g/L). FITC-IgY was purified from unconjugated FITC by collecting 1mL fractions from a 1.5cm x 30cm column of Sephacryl S-100 eluted with bicarbonate buffer. The fluorescein-labelled IgY was quantitated by assuming that all the IgY was coupled to FITC. Thus 16g/L was diluted by a factor of two when reacted with FITC to give 8g/L and 1mL (8mg) of this solution was loaded on to the Sephacryl S-100 column and collected in 2mL to give a final concentration of 4g/L.

2.1.3.2 Preparation of antibody:Sepharese solid phase

Ovine anti-chicken IgY serum and the non-specific serum (NSB) were coupled to CNBr activated 4 Fast Flow Sepharose following the manufacturer's instructions. Thus 1g of CNBr activated Sepharose, which gives about 3.5mL final gel volume, was washed, reswollen using 1mM HCl (200mL/g), centrifuged at 3,500xg for 5 minutes at 20°C and the supernatant discarded with each wash and swell cycle. A total of 0.5mL, 1mL and 2mL of serum batches 1013 and 1014 and of NSB serum were coupled respectively per gram of Sepharose. A sodium sulphate fractionation (18% (w/v)) with two washes was carried out on each of these serum batches to obtain partially purified IgG and 0.5mL, 1mL and 2mL sulphate fractions obtained from the serum batches (1013 and 1014) were coupled per gram of Sepharose. In addition 0.05mL, 0.1mL and 0.25mL of serum batch 1013 and of NSB serum were coupled respectively per gram of Sepharose. Each volume of serum or sodium sulphate fraction was diluted 1:2 in bicarbonate buffer (0.1M, pH 8.3) prior to coupling. End-over-end mixing was continued overnight at 4°C. and the remaining active groups blocked with 1M

ethanolamine for 2hr at room temperature. Excess protein and blocking agent were washed away employing alternate low (acetate buffer, 0.1M, pH4, containing 0.5M NaCl) and high (bicarbonate buffer, 0.1M, pH 8.3, containing 0.5M NaCl) pH cycles. Finally the protein-Sepharose conjugates were resuspended in twice their original volume of bicarbonate buffer (0.1M,pH 8.3) and stored at 4°C. ready for use. The coupling efficiency for each protein-Sepharose conjugate was calculated by carrying out an absorbance (280nm) reading of the starting material to be coupled and of the material remaining in the supernatant after coupling and calculating the percentage of the latter.

2.1.3.3 Anti-IgY dilution curves

Principles of dilution curves

Labelled ligand (fluorescein-labelled IgY in this example) assays involve two basic experimental procedures – antibody dilution curves and standard curves. The antibody dilution curve involves the incubation of a fixed amount of tracer ligand with different concentrations of the antibody; for example, serial doubling dilutions of an antiserum. Following incubation the distribution of the tracer in the bound and free fractions is ascertained. Given a suitable set of reagents, the construction of the dilution curve is the first step in setting up a binding assay system, since the result may determine the amount of antibody for use in a standard curve.

Using the solid phases described above, doubling dilutions of the anti-IgY solid-phase from approximately 17mg to 0.03mg of Sepharose gel were made in borate buffer (4.8g/L disodium tetraborate, pH 9, containing 1mL/L Triton X-100). To 100µL of each dilution was added 50µL borate buffer and 200µL of fluorescein-labelled chicken IgY (1.6µg IgY). The tubes were incubated for 30min at room temperature while mixing on a mechanical shaker. Volumes were made up to 1mL by addition of borate buffer and separation of the unbound fraction achieved by centrifugation for 5 minutes at 3,500rpm and 4°C, followed by aspiration of the supernatant. The bound fraction was eluted with 1.5mL of elution reagent

(0.1M sodium hydroxide, containing 1mL/L Triton X-100) for 10min at room temperature on a mechanical shaker and then centrifuged for 5 minutes at 3,500rpm and 4°C. Fluorescence intensities of the supernates were determined using a Perkin-Elmer LS-20 filter fluorimeter.

2.1.3.4 Standard curves

Principles of standard curves

The standard curve involves the incubation of fixed amounts of tracer ligand (fluorescein labelled IgY in this example) and antibody (determined from the dilution curve) with different concentrations of unlabelled ligand. When the sample is substituted for the standard, and using the same fixed concentrations of antibody and tracer, the value determined for the distribution of bound and free phases will be equivalent to some value on the horizontal scale of the standard curve. This value can be read by simple extrapolation. The standard curve is the basic requirement for quantitation of the ligand in unknown samples.

Protein-Sepharose conjugates were made with 0.5mL/g and 0.05mL/g for serum batch 1013 and 0.5mL/g for serum batch 1014.

A stock solution of chicken IgY was prepared using Sigma chicken IgY in saline (150mM NaCl) and determining the protein concentration by absorbance at 280nm with an extinction coefficient of 1.4 (1g/L solution, 1cm path length). The IgY standards (doubling dilutions of a 1g/L solution with a final standard concentration of 0.002g/L) were prepared by appropriately diluting the stock solution in borate buffer. To duplicate tubes containing 50µL of each standard was added 200µL (1.6µg IgY) of fluorescein-labelled IgY, followed by 100µL (2mg of 0.5mL serum per gram (batches 1013 and 1014) and 10mg of 0.05mL serum per gram of Sepharose solid phase (batch 1013)) of the anti-IgY sepharose solid-phase in borate buffer. Incubation, separation and fluorescence measurements were carried out as described above.

2.1.3.5 Precision

The precision profiles were constructed for standard curves employing 0.5mL and 0.05mL of serum (batch 1013) per gram of Sepharose solid phase (2mg and 10mg respectively of solid phase). The mean and standard deviation for ten replicates were used to calculate the coefficient of variation, which was plotted against the standard concentration.

All further standard curves were prepared using 10mg of solid phase prepared with 0.05mL of serum batch1013 per gram.

2.1.3.6 Incubation time

Standard curves were compared using incubation times of 30, 60, 120, 240 minutes and overnight.

All further standard curves were incubated for 120 minutes at room temperature.

2.1.3.7 Elution time

Standard curves were compared using immediate, 10 and 20 minute elution times.

2.1.3.8 IgY recovery

The removal of lipoproteins from chicken egg yolk was carried out using five different methods: PEG (Polson et al.1980), chloroform (Polson et al.1990), dextran sulphate (Jensenius et al. 1981), freezing and thawing (Jensenius et al. 1981) and caprylic acid (McLaren et al. 1994). These methods will be described in detail later in the project.

Samples produced by each method and chicken serum samples were assessed for their IgY content as above. Each sample was then diluted in assay buffer to give IgY concentrations close to 4mg/L. Exogenous chicken IgY (Sigma) was added to each sample to give a final concentration of 56mg/L following which the samples were reassayed.

2.1.3.9 Parallelism

Samples produced by each of the lipoprotein removal methods were diluted serially in assay buffer and the resulting curves compared with those of the standards.

2.1.3.10 Cross-reactivity with equine and ovine IgG

The cross reactivity of IgY from chickens was compared with that of ovine and of equine IgG. It was defined as the concentration of chicken IgY that produces 50% tracer bound, divided by the concentration of cross-reactant required to produce 50% binding.

2.1.3.11 Sensitivity

The sensitivity was calculated from ten replicates of the zero concentration of chicken IgY. The standard deviation of the signals from these replicates was used to estimate the minimal detectable concentration giving a 95% confidence level.

2.1.3.12 Assay application

The standard curve was used to estimate total IgY serum concentrations in immunized hens.

2.1.4 Results

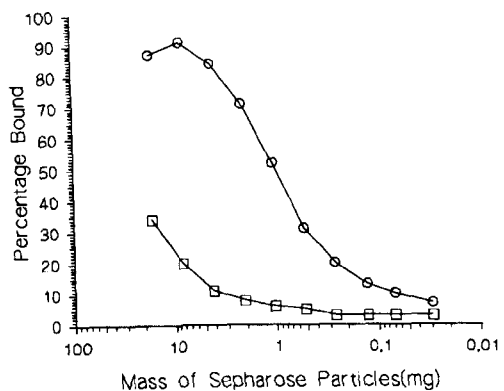
2.1.4.1 Preparation of fluorescein-labelled chicken IgY

The eluted peak corresponds to labelled chicken IgY.

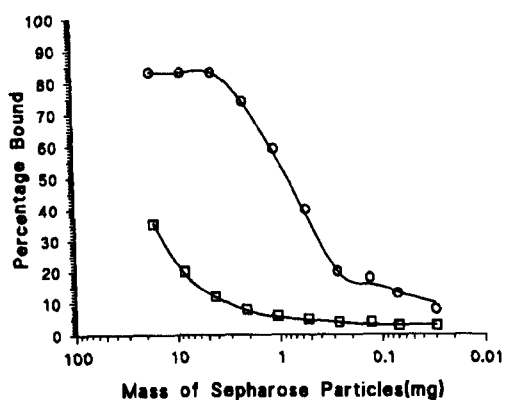
2.1.4.2 Anti-IgY dilution curves

Ovine anti-IgY sera from two different sheep were used to prepare antibody:Sephadex solid phases (Figure 2.1 and 2.2) and assessed by dilution curve. The purified IgG from serum sample 1013 was also evaluated (Figure 2.3). There was no significant difference between the dilution curves for the two different sheep serum samples and no advantage gained by using sodium sulphate-precipitated IgG.

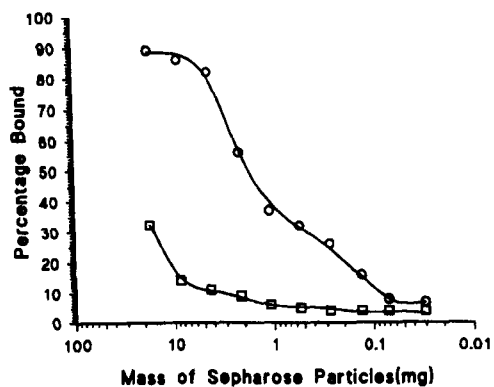
Based on the antibody dilution curves, approximately 10mg of Sephadex particles were chosen for the construction of the standard curve, which resulted in approximately 70% of the labelled IgY being bound in the absence of unlabelled IgY.



(a) 0.5mL serum per gram of Sepharose.

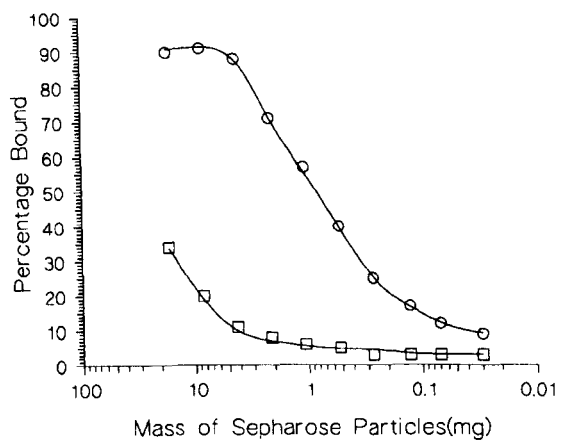


(b) 1mL serum per gram of Sepharose.

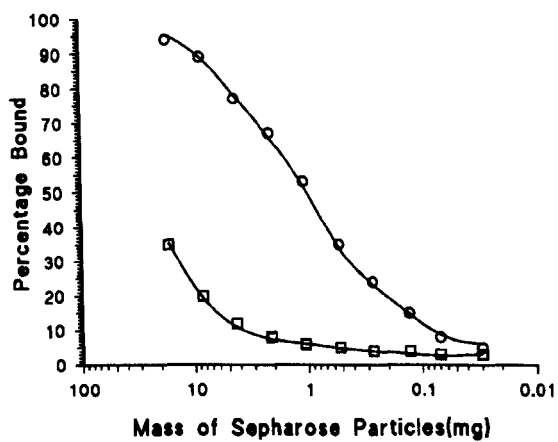


(c) 2mL serum per gram of Sepharose.

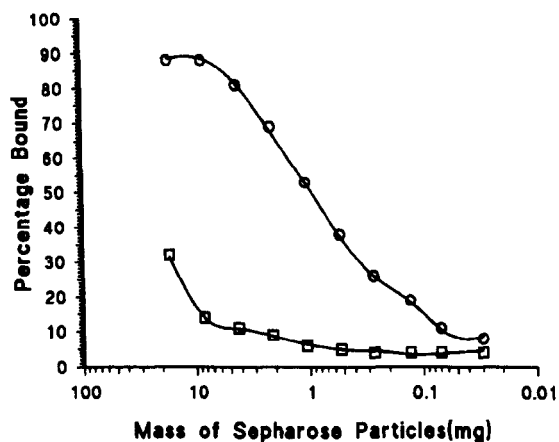
Figure 2.1 Assessment of the antibody:Sepharose solid phases prepared with serum batch 1013. Dilution curve(\circ), NSB (\square).



(a) 0.5mL serum per gram of Sepharose.

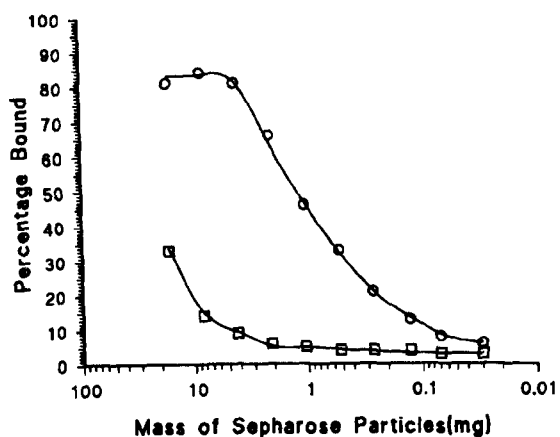


(b) 1mL serum per gram of Sepharose.

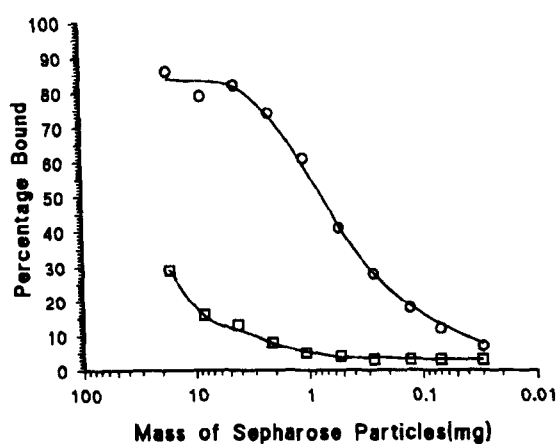


(c) 2mL serum per gram of Sepharose.

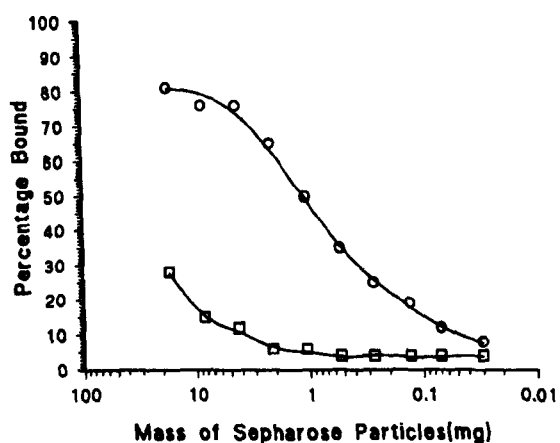
Figure 2.2 Assessment of the antibody Sepharose solid phases prepared with serum batch 1014. Dilution curve(\circ), NSB (\square).



(a) 0.5mL sodium sulphate fraction per gram of Sepharose.



(b) 1mL sodium sulphate fraction per gram of Sepharose.



(c) 2mL sodium sulphate fraction per gram of Sepharose.

Figure 2.3 Assessment of the antibody:Sepharose solid phases prepared with sodium sulphate fraction of serum batch 1013. Dilution curve(○), NSB (□).

2.1.4.3 Standard curves

The standard curves covered a range of IgY concentrations from approximately 10mg/L to 2000mg/L (Figure 2.4 a and b).

2.1.4.4 Precision

The within assay coefficient of variation ($\%CV = \text{standard deviation} / \text{mean} \times 100$) calculated from the results of ten replicates for each standard was less than 10% covering the range of values from 30mg/L to 800mg/L (Figure 2.5 b) for a standard curve constructed with 10mg of solid phase.

2.1.4.5 Incubation and elution times for standard curve

Incubation for 2hrs (Figure 2.6a) and elution for 10 minutes (Figure 2.6b) was satisfactory.

2.1.4.6 Analytical recovery

Recoveries of IgY, when added to give exogenous IgY concentrations of 56mg/L in yolk extracts prepared by freezing and thawing, dextran sulphate, caprylic acid, chloroform and PEG lipoprotein were 98%, 112%, 97%, 97% and 98% respectively. For chicken serum the recovery was 96%.

2.1.4.7 Parallelism

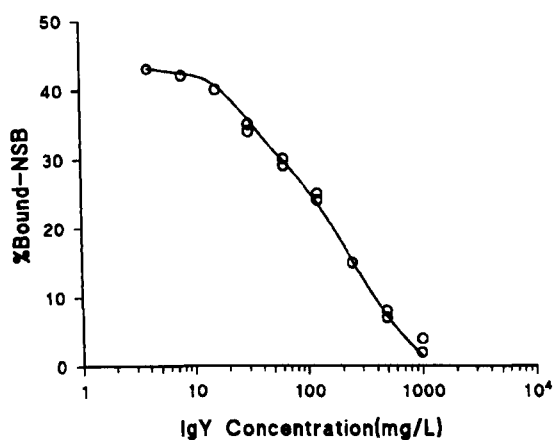
When diluted serially in assay buffer, yolk extracts obtained by caprylic acid, dextran sulphate, chloroform, freezing and thawing and polyethylene glycol gave curves paralleling those of the standards (Figure 2.7a). A chicken serum sample diluted serially in assay buffer also gave a curve with values paralleling those of the standards (Figure 2.7b).

2.1.4.8 Cross-reactivity with ovine and equine IgG's

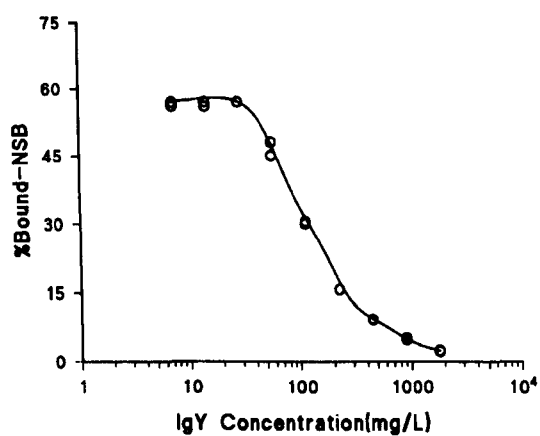
When tested at a concentration of 1g/l there was no detectable immunoreactivity (Figure 2.8).

2.1.4.9 Sensitivity

Sensitivity was calculated from the mean signal of ten replicates of the zero concentration for IgY and the standard deviation. The mean minus two standard deviations was used to

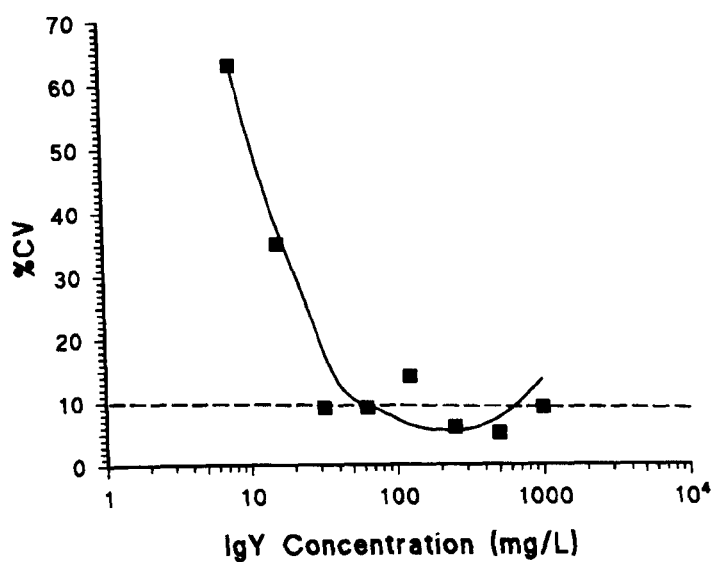


(a) 2mg of solid phase prepared with 0.5mL serum (batch 1013) per gram of Sepahrose.

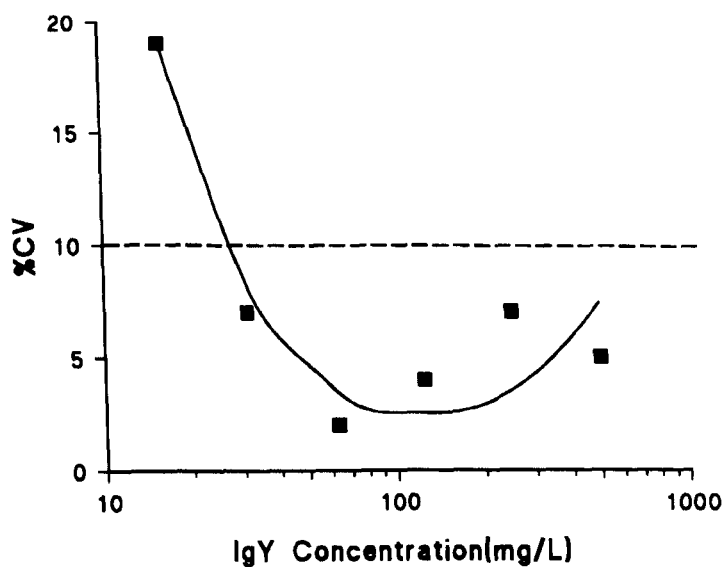


(b) 10mg of solid phase prepared with 0.05mL serum (batch 1013) per gram of Sepahrose.

Figure 2.4 Standard curves for chicken IgY in serum (○).

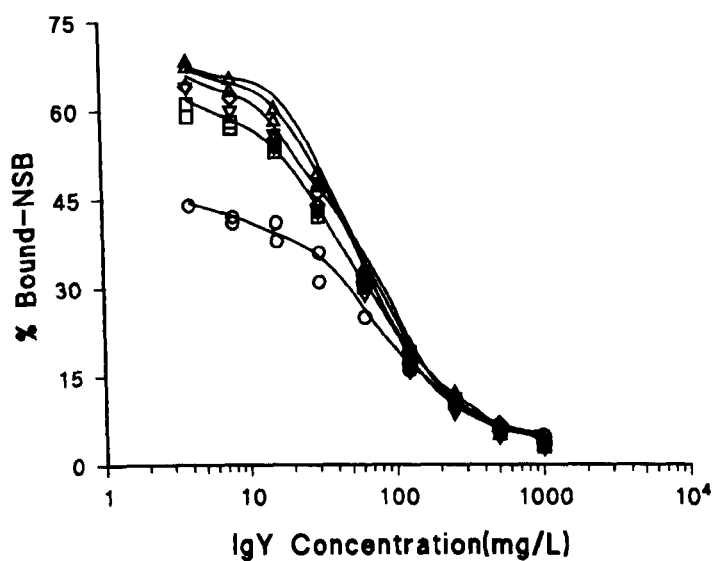


(a) 2mg of solid phase prepared with 0.5mL serum (batch 1013) per gram of Sepharose.

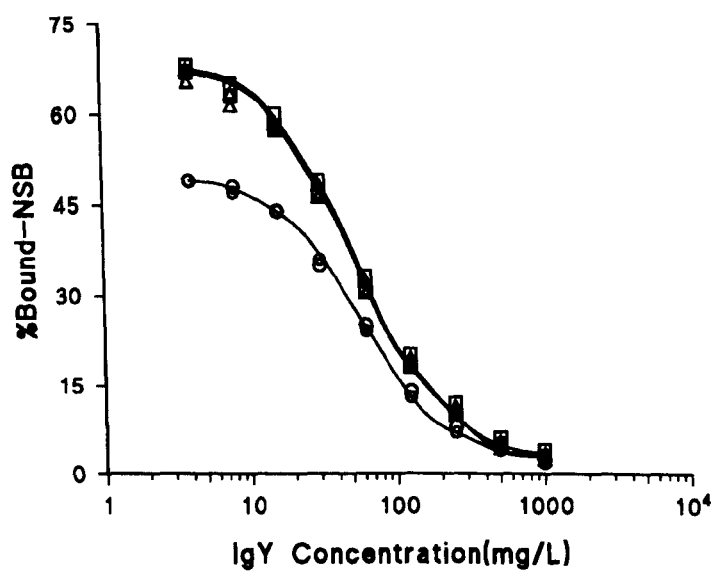


(b) 10mg of solid phase prepared with 0.05mL serum (batch 1013) per gram of Sepharose.

Figure 2.5 Within assay percentage coefficients of variation were calculated from the results of ten replicates for each standard.

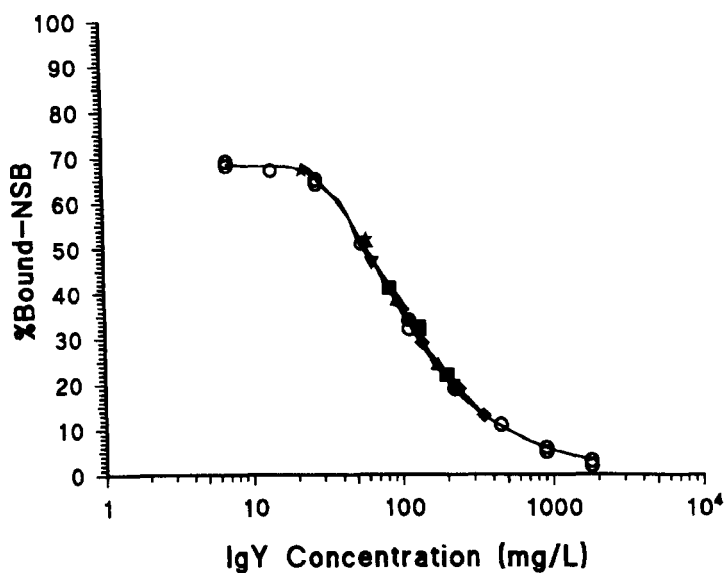


(a) Incubation time in minutes: 30min (○), 60min (□), 120min (Δ), 240min (▽) and overnight (◇).

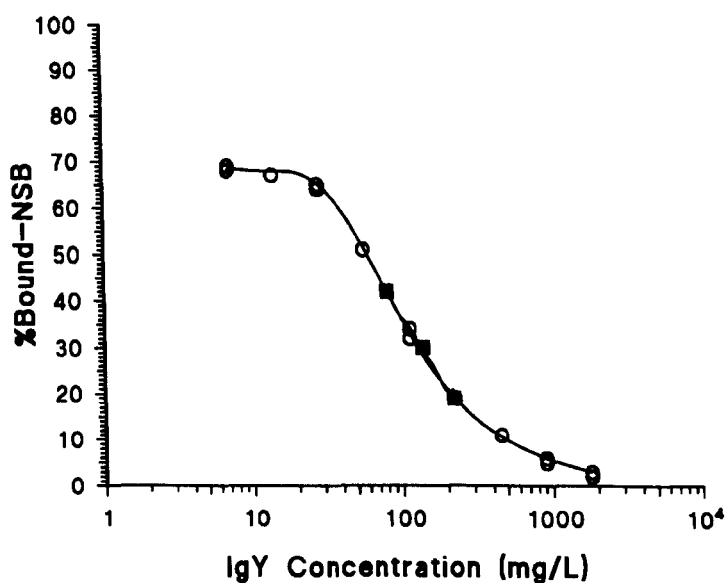


(b) Elution time in minutes: Immediate (○), 10min (□) and 20min (Δ).

Figure 2.6 Incubation and elution studies



(a) Samples produced from five lipoprotein removal methods: Caprylic acid(♦), chloroform(■), polyethylene glycol(▲), dextran sulphate(▼), and freezing and thawing(★) .



(b) Chicken serum sample(■).

Figure 2.7 Samples, all serially diluted in assay buffer, and the resulting curves compared against that of the standards(○).

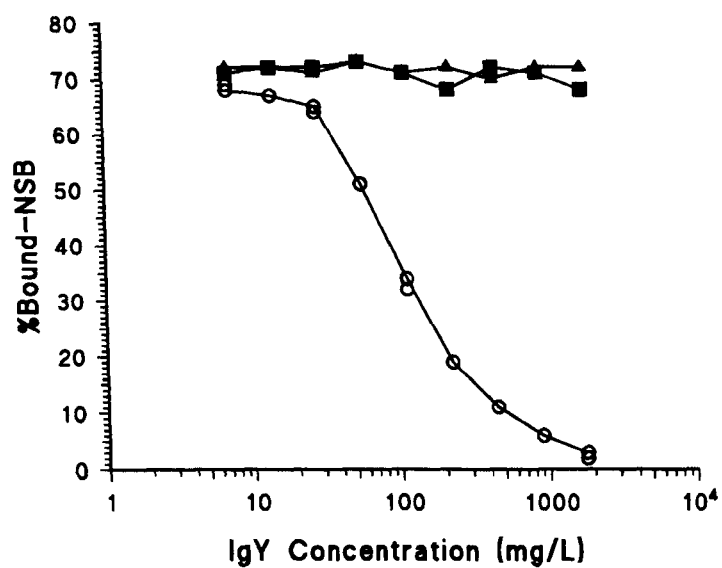


Figure 2.8 Cross reactivity of chicken serum IgY (○) compared with ovine IgG(■) and equine IgG(▲).

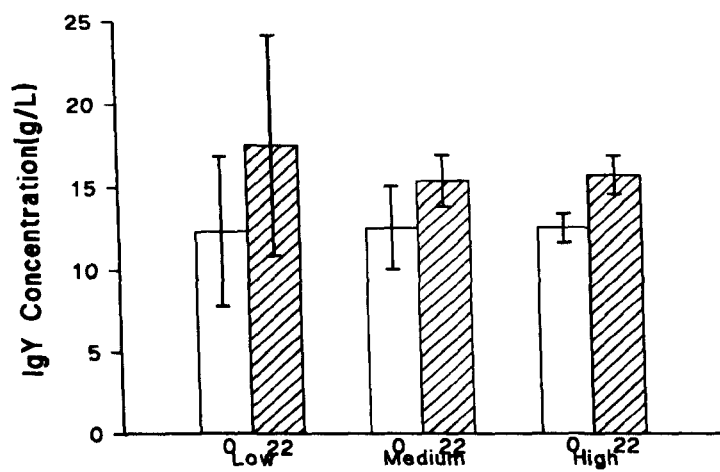


Figure 2.9 Application of the IgY immunoassay to estimate total serum IgY concentrations in immunised hens. Preimmune and 22 week serum samples in hens (n=3) immunised with snake venom:adjuvant emulsion

estimate the minimal detectable concentration as 15mg/L .

2.1.4.10 Assay application

There was no major difference in IgY serum concentrations between preimmune and 22 week immunised hens for chicken groups (n=3) immunised with low and medium venom doses. There may be a difference, albeit small, for chickens immunised with the high venom dose (Figure 2.9).

2.1.5 Discussion

Dilution curves of two ovine anti IgY sera were similar with regards to non-specific and maximum binding and specific antibody titre. No advantage was gained by purifying the ovine immunoglobulins prior to use. Standard curves constructed using 2mg of solid phase prepared by coupling 2mL of each serum per gram of solid phase were also similar. Serum batch 1013 was chosen for further work because larger stocks were available. The precision profile using 0.5mL of antisera per gram of Sepharose (Figure 2.5) was acceptable (less than 10%) only over a narrow range. This was attributed to the unintentional loss of some particles during aspiration and it was decided to increase the mass of solid phase used without altering the volume of the reactants. This was achieved by coupling only 0.05mL serum per gram of solid phase and using 10mg of solid phase (five times that used previously). The coefficient of variation was in the acceptable range (of less than 10%) over a wider range which extended from approximately 20 to 800mg/L.

With the small numbers of hens studied there was no significant difference in total IgY serum concentrations before and after a 22 week course of immunisation of hens with low and medium venom doses. There might be some difference albeit small in the chicken group immunized with the high venom dose.

The fluoroimmunoassay described above offers several advantages. These include sensitivity and specificity sufficient for all analytical tasks under consideration and results are obtained on the same day with good precision.

2.2 Simple assessment methods: Characterisation of a small scale affinity assay for determining specific antibody concentrations

2.2.1 Background

Since Theakston et al. (1977) described the use of the enzyme-linked immunosorbent assay (ELISA) to detect venom antigen and antibody in snakebite patients, this technique has been applied widely to the study of snake bite and assessment of antivenoms.

Ho et al. (1986) argued that the ELISA technique, as described by Theakston et al. (1977), had been applied (Coulter et al. 1980; Theakston et al. 1981 a, b) without properly defining important parameters such as sensitivity and specificity based upon studying non-specific and cross reactivity. As a result, many of the published earlier findings and conclusions were open to question. They concluded that venom antibody detection ELISAs were at a much less satisfactory stage of development than those for antigen and that their role in screening of antivenoms was doubtful unless defined toxic venom components were used as antigen.

In our laboratory, we employ manual ELISA in the screening of the humoral immune response in different host animals, using the host's pre-immunisation sample as its own control, thus following one of Ho et al. (1986) recommendations. Cross reactivity is not a problem in this application but we have encountered another difficulty with ELISA not mentioned by Ho et al. (1986), namely marked imprecision. This may be due to the fact that ELISA has multiple steps and the reactants involved in each step may not come to equilibrium before moving on to the next step. Such assays are also extremely sensitive to temperature fluctuations. Despite this, our ELISA method has been useful in screening large numbers of animals being immunised with venoms.

The aim of this work is to characterise a procedure based on small scale affinity chromatography as an alternative to the ELISA method currently in use. The method is simpler, more precise and enables the actual physical quantities of the therapeutic

antibodies in host animals to be determined in g/L (as opposed to the 50% binding titre results obtained from ELISA). It also requires less expensive equipment so providing a simple, inexpensive method suitable for use in developing countries. Such a procedure was described by Smith et al. (1992), but no development and optimisation studies were included. Studies with *V.latastei* venom are used as a model.

2.2.2 Materials

Cyanogen bromide activated 4 Fast Flow Sepharose (CNBr) was obtained from Amersham Pharmacia Biotech; *V. latastei* venom was donated by Dr. Delfin Gonzalez, Spain and ovine donor sera by MicroPharm Ltd. Glass columns (1 x 10cm) were from BioRad and all other chemicals from Sigma.

2.2.3 Methods

2.2.3.1 Preparation of venom:Sepharose solid phase

One gram of CNBR activated 4 Fast Flow Sepharose (which gives about 3.5mL final gel volume) was washed and reswollen using 1mM HCl (200mL/g), and centrifuged at 3,500xg for 5 minutes at 20°C. This was repeated and the supernatant discarded with each wash and swell cycle. *V. latastei* venom was then coupled to the sepharose following the manufacturer's instructions.

A 2g/L venom solution was prepared in bicarbonate buffer (0.1M, pH8.3). The coupling reactions were carried out by incubating 0.5mL, 2.5mL and 5mL (1mg, 5mg and 10mg) respectively of the venom solution with the activated Sepharose while 5mL of bicarbonate buffer was incubated with Sepharose to prepare a control matrix.

End-over-end mixing was continued overnight at 4°C, and the remaining active groups blocked with 1M ethanolamine for 2hr at room temperature. Excess protein and blocking agent were washed away alternately with acetate buffer (0.1M, pH4 containing 0.5M NaCl) and bicarbonate buffer (0.1M, pH8.3 containing 0.5M NaCl). Finally the venom:Sepharose conjugates were resuspended in twice their original volume of phosphate buffered saline

(PBS), poured into a glass column (1x10cm) and stored at 4°C ready for use. The coupling efficiency for each venom:Sephadex conjugate was calculated by reading the absorbance (280nm) of the starting material to be coupled and of the material remaining in the supernatant after coupling.

A total of 1mg, 5mg and 10mg of *V. latastei* venom were coupled in triplicate per gram of Sephadex. In addition, ten replicates of 5mg of *V. latastei* venom were coupled per gram of Sephadex.

2.2.3.2 Small-scale affinity purification (SSAP)

All procedures were performed at ambient room temperature as described below, unless otherwise stated.

The SSAP columns were removed from the refrigerator, placed in a suitable rack and allowed to equilibrate to room temperature and then washed with 25mL of washing buffer (10mM sodium phosphate buffer, pH 7.5, containing 9g/L sodium chloride). Each column was allowed to drain until there was no liquid on top of the venom:Sephadex matrix.

All antiserum samples were centrifuged (20 minutes, 3500rpm, 25°C) before typically adding 0.5mL to the SSAP column. Assuming that the bed volume of the column was 3.5mL, sufficient washing buffer was added to make the total volume in each column up to 10mL. Each column was capped and the contents mixed on an end-over-end mixer for 1h at room temperature. The columns were returned to the rack, the gel matrix allowed to settle, and they were then drained until there was no liquid on top of the matrix. Next each column was washed with 25mL of washing buffer and allowed to drain until there was no liquid on top of the matrix.

Finally 20mL of elution buffer (100mM glycine/HCL, pH 2.5) (This elution buffer has been used in-house satisfactorily for other applications) was applied to each column to elute the bound specific antibodies and the entire eluate (20mL) was collected and set aside for

optical density measurement. After use, each column was washed with 25mL of washing buffer and then stored at 2 - 8°C.

Using a UV spectrophotometer and cuvettes with 1cm path length, the optical density (OD) at 280nm of each column eluate was measured using elution buffer to blank the spectrophotometer. The concentration of specific antibodies in the antiserum sample was calculated using the following equation:

$$\text{Concentration in sample} = (\text{OD}/\text{E}) \times (20/\text{V}).$$

Where E is the extinction coefficient for ovine IgG (1.5) (Curd et al. (1971)) and V is the volume of antiserum sample added to the affinity column.

2.2.3.3 Capacity determination of venom: Sepharose column matrices

A total of 0.25, 0.5, 1, 2, 4, and 8mL of ovine *V. latastei* antisera containing high concentrations of specific antibodies and of donor sera respectively were added to each previously prepared Sepharose matrix coupled with 0, 1, 5 and 10mg of *V. latastei* venom per gram of Sepharose. The serum samples were assessed following the SSAP procedure described above.

2.2.3.4 Between and within column variation of replicate matrices

2.2.3.4.1 Between column variation

0.5mL of *V. latastei* antisera was added to each matrix and the serum samples assessed following the SSAP procedure described elsewhere (See section 2.2.3.2 ,p.70).

2.2.3.4.2 Within column variation

Seventy four SSAP procedures have been carried out on the same matrix with the addition of 0.5mL of *V. latastei* antisera. A SSAP procedure with addition of 0.5mL donor serum was carried out after the first SSAP with *V. latastei* antisera and this was repeated after the seventy-fourth procedure in order to monitor non-specific binding (NSB).

2.2.3.5 Identity of eluate

Six SSAP procedures were carried out with the addition of 0.5mL of *V. latastei* antisera. The eluate was collected in 1mL x 20 fractions for each procedure and an optical density measurement carried out for each 1mL fraction. The fractions for each procedure (typically 3mL) contributing to approximately seventy five per cent of the total optical density were pooled and dialysed over night in 10L washing buffer(10mM sodium phosphate buffer, pH 7.5, containing 9g/L sodium chloride). The dialysed samples were then assessed by SDS-PAGE as described elsewhere (See section 3.1.3.3.3, p87).

2.2.3.6 Analytical recovery

The dialysed samples from the six runs produced in the “Identity of eluate” study were pooled and their optical density (280nm) estimated. Two mL of sample (concentration x g/L specific IgG) was added to 2mL of donor serum and assuming the bed column volume was 3.5mL, 2.5mL washing buffer was added to make the total volume in each column up to 10mL. The samples were then processed following the SSAP described elsewhere (See section 2.2.3.2 ,p.70). This experiment was carried out in triplicate. The analytical recovery (AR) was calculated using the following equation:

$$AR = ((\text{Total measured (mg)} - 1 \text{ mg of NSB})/\text{Total added (mg)}) \times 100.$$

2.2.3.7 Incubation time

SSAP procedures were compared using incubation times of 0, 15, 30, 60, 120, 240 min and overnight.

2.2.3.8 Wash volume

SSAP procedures were compared using wash volumes of 12.5, 25, 50 and 100mL. The experiments were repeated with donor serum.

2.2.3.9 Assay application

All SSAP procedures henceforth were carried out employing a one hour incubation time at room temperature and a 25mL wash volume.

The SSAP procedure was used to estimate the dose response of sheep immunized with progressively lower doses of venom of *V. latastei* (Table 2.1) at four week intervals over forty four weeks by MicroPharm Ltd. The sheep were sampled two weeks after every immunisation.

2.2.4 Results

2.2.4.1 Coupling efficiency

This was calculated as being greater than 80% for all venom:Sephadex conjugates prepared.

2.2.4.2 Capacity determination

The 1mg/g matrix was sufficient for volume loads of specific antisera of 0.25 and 0.5mL. Thereafter its capacity for specific antibodies began to be saturated. The 5mg/g and 10mg/g matrix were sufficient for volume loads of up to 1mL but began to be saturated when larger volumes were added. All matrices inclusive of the 0mg/g demonstrated non-specific binding corresponding to a protein concentration in the sample (employing extinction coefficient 1.5) of 1g/L or less (Figure 2.10a). All SSAP procedures henceforth were carried out using a 5mg/g Sephadex column matrix with a 0.5mL serum sample load.

2.2.4.3 Between column variation

The coefficient of variation ($\%CV = \text{standard deviation} / \text{mean} \times 100$) of the concentration in the sample (g/L) as calculated from ten replicates was less than 5% (Figure 2.11a). The non-specific binding characteristics of the solid phase calculated from ten replicates with coupled venom and donor serum was 1g/L or less (Figure 2.11b).

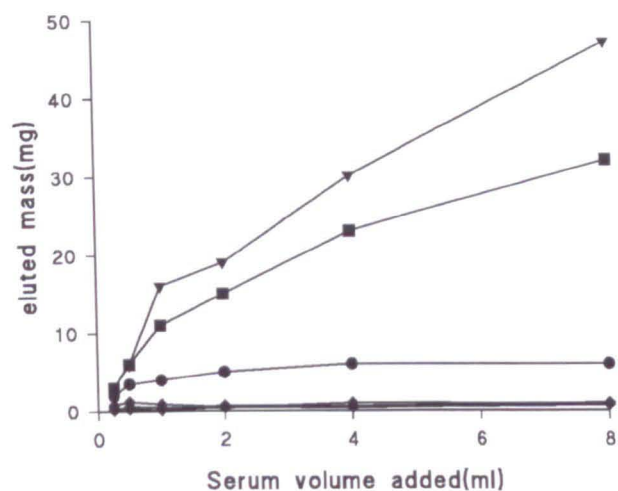
2.2.4.4 Within column variation

The coefficient of variation from seventy four replicates was approximately 6% (Figure 2.11c). There was little or no evidence of decline in result with time. However, these studies will be extended to assess column useful lifetime over one hundred runs.

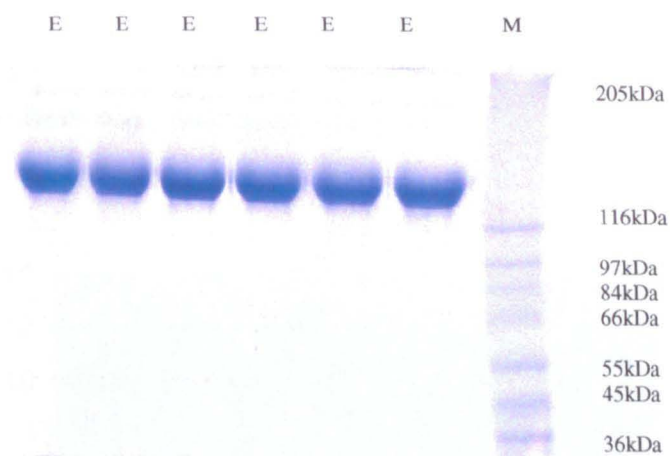
2.2.4.5 Identity of eluate

Immunisation/ Sample	Weeks post primary immunisation	Immunogen Dose (mg) per sheep						
		Group I (6 sheep)	Group II (6 sheep)	Group III (6 sheep)	Group IV (6 sheep)	Group V (3 sheep)	Group VI (3 sheep)	Group VII (3 sheep)
Primary immunisation	0	0.015625	0.03125	0.0625	0.125	0.25	0.5	0.5
Re- immunisation	4	0.015625	0.03125	0.0625	0.125	0.25	1	1
Sample	6							
Re- immunisation	8	0.015625	0.03125	0.0625	0.125	0.25	1	2
Sample	10							
Re- immunisation	12	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	14							
Re- immunisation	16	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	18							
Re- immunisation	20	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	22							
Re- immunisation	24	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	26							
Re- immunisation	28	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	30							
Re- immunisation	32	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	34							
Re- immunisation	36	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	38							
Re- immunisation	40	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	42							
Re- immunisation	44	0.015625	0.03125	0.0625	0.125	0.25	1	4
Sample	46							

Table 2.1 Immunisation protocol for sheep dose response study

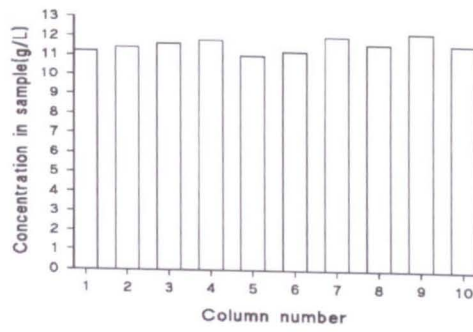


(a) 0mg/g (◆), 1mg/g (●), 5mg/g (■), 10mg/g (▼) assessments with specific antisera. All donor serum assessments of matrices 1, 5, and 10mg/g are also represented by the symbol (◆).

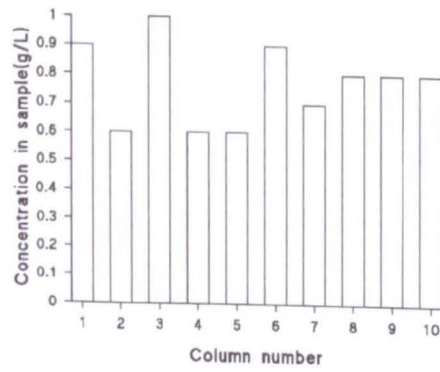


(b) Six replicate eluates (E) and molecular weight markers.

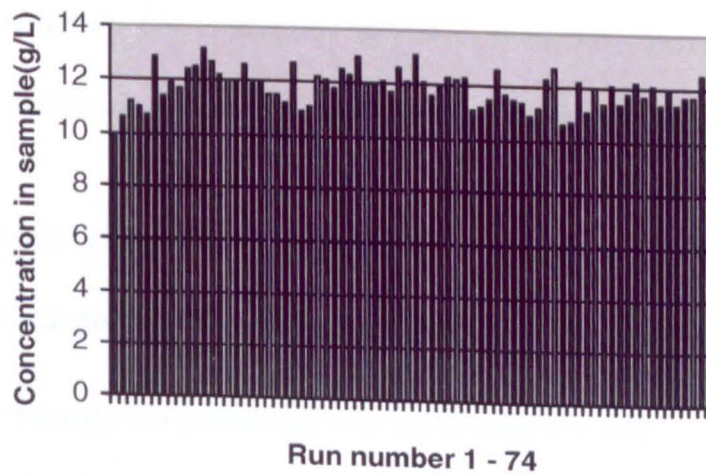
Figure 2.10 Capacity determination of venom-coupled Sepharose matrices(a) and eluate identity assessment(b).



(a) Between column variation with specific antisera



(b) Between column variation with donor serum



(c) Within column variation with specific antisera

Figure 2.11 Assessment of between and within column variation of replicate matrices

SDS-PAGE assessment of the eluate of replicate SSAP procedures resulted in a dominant single band corresponding to the molecular weight of IgG (Figure 2.10(b)).

2.2.4.6 Analytical recovery

The mean recovery of specific IgG when added to donor serum was 74%.

2.2.4.7 Incubation time

An incubation time of 30 to 60 min at room temperature was satisfactory (Figure 2.12(a)).

2.2.4.8 Wash volume

Approximately seven column volumes (25mL) was found to be satisfactory (Figure 2.12(b) and (c)).

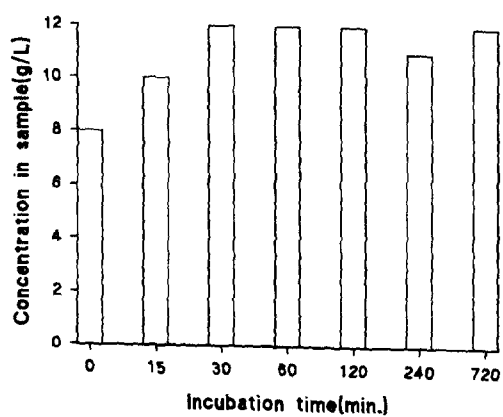
2.2.4.9 Assay application

After the first immunisation of 0.5mg of venom per sheep in group seven the mean specific antibody concentrations were estimated at 7g/L. This concentration increased with two subsequent immunisations of 1 and 2mg of venom to means of 10g/L (Figure 2.13(a)). A further eight consecutive immunisations with 4mg of venom resulted in a mean of 12g/L.

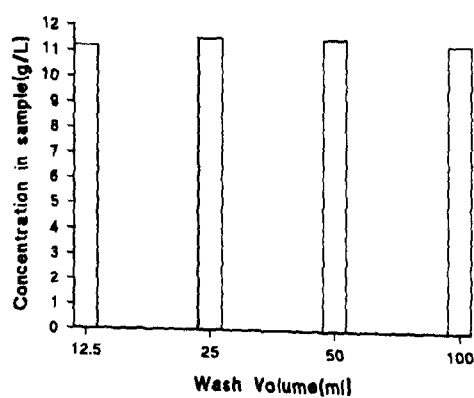
Overall, the specific antibody concentrations increase with subsequent dosing and then reach a maximum and plateau in sheep in all venom dose groups (Figure 2.13(b)).

2.2.5 Discussion

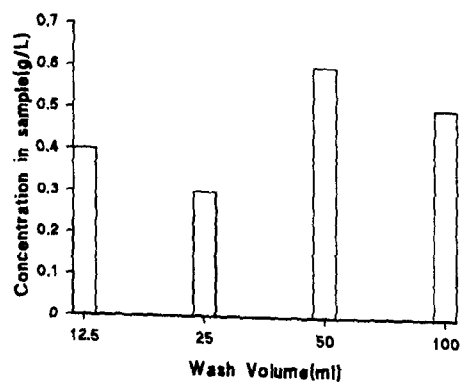
The venom:Sepharose solid phase prepared with 5mg of venom per gram of Sepharose was considered the most satisfactory for the analytical tasks under consideration and enabled an economical use of the venom. No advantage was gained from using a solid phase coupled with 10mg of venom. Furthermore, there may be a limit to the amount of specific antibodies that can bind to the solid phase due to steric hindrance or interference; thus increasing the amount of coupled venom may not result in more antibody binding. This



(a) Incubation time comparison

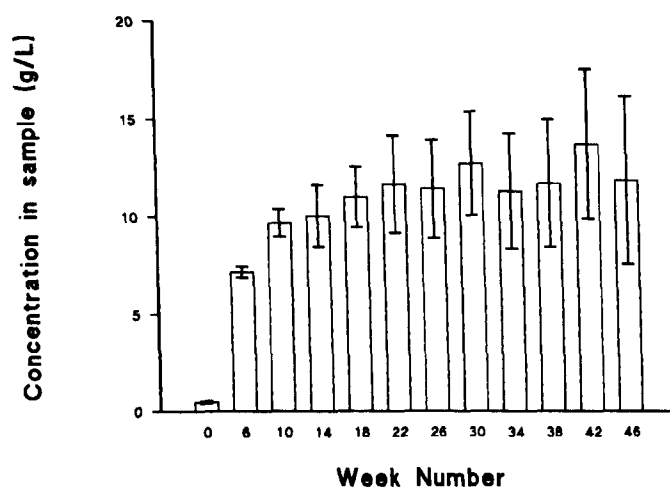


(b) Wash volume comparison with specific antisera

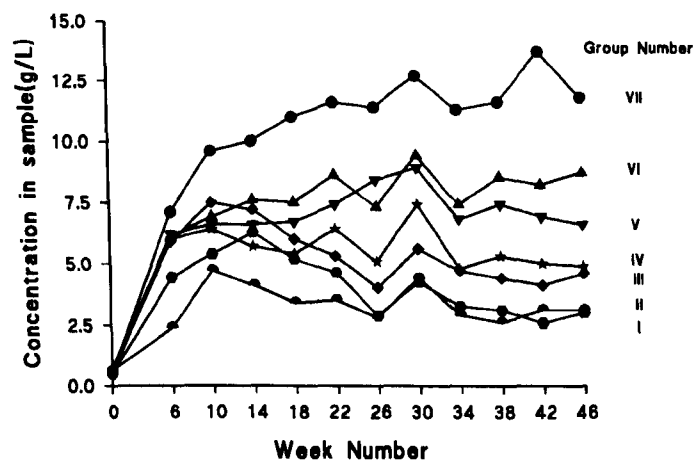


(c) Wash volume comparison with donor antisera

Figure 2.12 Assessment of incubation time and wash volume



(a) Example of specific antibody concentrations in group VII sheep



(b) Summary of mean specific antibody concentrations in all dose groups

Figure 2.13 Assessment of specific antibody concentrations over time

observation needs further investigation.

The non-specific binding characteristics of the solid phase with and without coupled venom were similar at 1g/L or less. This suggests that the Sepharose matrix was responsible for the observed non-specific binding and that the coupled venom even at a concentration of 10mg/g of Sepharose was not a contributing factor. It has to be emphasized that the value for non specific binding determined here assumes a worst case scenario as it is determined using donor serum from a sheep that was been immunised with venom. If increased accuracy and reliability were requirements then it may be necessary to investigate strategies for reducing non-specific binding, using donor serum as a model.

Confirmation that the eluate is mainly IgG by SDS-PAGE increases confidence in the use of optical density at 280nm for determining the concentration of specific antibodies.

Sheep immunized with subsequent 4mg doses of venom (group VII) produce the highest concentrations of specific antibodies reaching a maximum and mean plateau of approximately 12g/L. In view of the latter we have chosen a 4mg venom dose for future immunization programmes.

Sheep in the lowest venom dose group (group I) were still able to produce specific antibodies with a mean plateau of 3g/L. It will be interesting in a future study to investigate the concentration of specific antibodies in sheep immunised with venom doses lower than 16µg and include a control group of sheep immunized with adjuvant only.

The low coefficient of variation between columns (approximately 5%) suggests that we can estimate the between animal variation in their capacity to produce specific antibodies to the venom at around 19% (results calculated from sheep in group VII).

The SSAP procedure above offers several advantages. These include sensitivity, analytical recovery and longevity sufficient for all analytical tasks under consideration and results are obtained on the same day with good precision.

This work illustrates how SSAP can be used for the assessment of antisera; furthermore this analytical technique could be employed for the assessment of processing strategies for antisera to produce antivenoms.

CHAPTER 3: PREPARATION OF A BROAD SPECIFICITY ANTIVENOM FOR EUROPEAN VIPERS FROM YOLK IgY

3.1 Improved extraction and purification techniques for yolk

3.1.1 Background

Until the 1950's, preparative procedures for egg lipoprotein involved their precipitation from salt solutions by dilution, and extraction of the protein solution with ethyl ether to remove free lipid. Lea and Hawke (1952) had recognized that the solubility of the egg lipoproteins was seriously altered by freezing, drying and treatments that removed the bound fat and advised that all physical measurements be made on freshly prepared samples. Vandegaer (1956) pointed out that extraction with ethyl ether also changed the solubility of the lipoprotein fractions and recommended high-speed centrifugation rather than ether extraction to remove free lipid. During this period, investigators were trying to identify the different components of egg yolk and emphasis was placed on extraction strategies that had the least possible effect on samples that were going to be subject to subsequent analytical investigation.

Once Williams (1962) had established yolk as a source of antibodies, researchers were further stimulated to investigate extraction methods that were cost efficient, simple, rapid, easily scaled up and preserved the function of the globulin fraction.

Jensenius et al. (1981) pointed out that earlier published methods had limited value since they included extraction with organic solvents and ultracentrifugation. Subsequently, attempts were made to develop more efficient and convenient methods. The most promising of these involved precipitation of lipoprotein with polyethylene glycol (Polson and von Wechmar 1980), dextran sulphate (Jensenius et al.1981) or caprylic acid (McLaren et al. 1994) and dilution and freeze-thawing (Svendsen et al.1995). However it is difficult to compare results since the yield and purity of the isolated IgY are not always given and different methods were used to measure the activity of IgY.

In order to compare these methods, a triglyceride enzymatic assay (McGowan et al.1983) was employed to monitor lipid removal, an immunoassay for IgY to determine yield, SDS-PAGE to assess purity and ELISA to monitor activity.

3.1.2 Materials

Fluorescein isothiocyanate (FITC) isomer I and chicken immunoglobulin (IgY) were from Sigma; ovine anti-chicken IgY and an ovine non-specific binding control (NSB) from MicroPharm Ltd; cyanogen bromide (CNBr) activated 4 Fast Flow Sepharose from Amersham Pharmacia Biotech; chicken eggs (medium/class A) from Lion (eggs from caged hens chosen in order to minimize between egg yolk variation); egg yolk and serum from hens immunised with a mixture of European viper venoms (*V. berus*, *V. ammodytes* and *V. aspis*) from MicroPharm Ltd; chloroform and caprylic (N-Octanoic) acid from BDH; centrifuge tubes from Greiner Labortechnik; glass fibre prefilters from Sartorius; disposable cuvettes from Kartell (Part No.1941); Triglyceride Reagents A (Sigma, 337-40A) and B (Sigma337-10B) and glycerol standard; 250mg/dL (Sigma G 1394); molecular weight markers from Sigma; polystyrene 96-well micro-titre plates from Nalge Nunc International; and horseradish peroxidase labelled sheep anti-chicken IgG from MicroPharm Ltd. Dextran sulphate (average molecular weight of 500,000) from Sigma. All other chemicals were obtained from Sigma.

3.1.3 Methods

3.1.3.1 Preparation of egg yolk

The white was separated from the yolk using an egg separator taking care not to break the yolk. Each yolk was washed with 3 x 50mL saline and dropped into a measuring cylinder and its volume recorded (average 15mL). The yolks from a dozen eggs were pooled and half was frozen at -20°C overnight while the other half was stored at 4°C until needed. All subsequent work was carried out using 5mL aliquots of the pool and the results scaled up to 15mL, representing the average yolk volume.

3.1.3.2 Purification of IgY from egg yolk

On the basis of available knowledge purification was divided into two distinct steps namely lipoprotein removal (Step 1) and protein separation (Step 2). Several methods were compared.

3.1.3.2.1 Caprylic acid and ammonium sulphate (McLaren et al.1994)

Ten mL of acetate buffer (0.06M, pH4) was added to 5mL of egg yolk and the pH adjusted to 4.8 by dropwise addition of 0.2M NaOH. Then 304 μ l of caprylic acid per mL of yolk was added dropwise with vigorous mixing, which was continued for one hour followed by centrifugation at 2750xg for one hour at 20°C. This produced an infranatant aqueous layer, with the lipid mass on its surface. Thus centrifugation was carried out in plastic tubes and a needle was used to pierce the latter and withdraw the aqueous layer leaving the lipoprotein mass behind (See Figure 5.1(a) p.140). The infranatant was filtered through a glass fibre filter to remove egg yolk sac debris, its pH adjusted to 7.5 with 1M Tris pH 8 and solid ammonium sulphate added to a concentration of 1.75M. After stirring at 4°C for one hour followed by centrifugation for 10 minutes at 2750xg and 25°C the supernatant was discarded and the pellet washed by resuspending in 1.75M ammonium sulphate to a volume equal to that of the undiluted harvested egg yolk and recentrifuged. A second wash with 1.75M ammonium sulphate was carried out and the washed pellet was then dissolved in PBS pH 7.5 to a volume equal to that of the undiluted harvested egg yolk.

3.1.3.2.2 Chloroform and polyethylene glycol (Polson, 1990)

Egg yolk was diluted 1:3 in 10mM phosphate buffer pH 7.5 containing 0.1M NaCl and two volumes of chloroform added. After mixing for one hour on a roller at room temperature and centrifugation at 2,750xg for 1hour at 20°C, the supernatant was removed to a fresh tube and solid polyethylene glycol 6000 added to a final concentration of 12% (w/v). The latter was mixed for one hour on a roller at room temperature, centrifuged at 2,750xg for 1

hour at 20°C, the supernatant discarded and the pellet dissolved in PBS pH 7.5 to a volume equal to that of the undiluted harvested egg yolk.

3.1.3.2.3 Dextran sulphate and sodium sulphate (Jensenius et al. 1981)

Egg yolk was mixed thoroughly with four times its volume of saline and 100µL of a 10% (w/v) dextran sulphate solution and 300µL of 1M calcium chloride added per mL of yolk/saline suspension. After mixing for one hour and then centrifugation at 2750xg for 1 hour at 20°C, the supernatant was decanted, dialysed against 5L of PBS pH 7.5. Solid sodium sulphate (20g per100mL) was added, mixed on a roller for half an hour at room temperature, centrifuged at 2750xg at 25°C and the supernatant discarded. The precipitate was washed twice by resuspension in sodium sulphate (20g per100mL) to a volume equal to that of the undiluted harvested egg yolk and recentrifuged. The washed pellet was dissolved in PBS pH 7.5 to a volume equal to that of the undiluted harvested egg yolk.

3.1.3.2.4 Dilution, freezing and thawing and sodium sulphate (Svendsen et al. 1995)

Egg yolk was diluted 1:10 in distilled water and the pH corrected to pH 7 using 0.1M NaOH. The diluted egg yolk was frozen for 24 hours at -20°C and then thawed at room temperature and centrifuged at 2,750xg for 1 hour at 4°C. Solid sodium sulphate (20g/100mL) was added to the supernatant, mixed on a roller for half an hour at room temperature and centrifuged at 2750xg. The supernatant was discarded and the precipitate resuspended in 20g/100mL sodium sulphate to a volume equal to that of the undiluted harvested egg yolk and recentrifuged. Two washes with sodium sulphate were carried out. The washed pellet was dissolved in PBS pH 7.5 to a volume equal to that of the undiluted harvested egg yolk.

3.1.3.2.5 Polyethylene glycol (PEG) (Polson et al. 1980)

Egg yolk was diluted 1:3 in 10mM phosphate buffer pH 7.5 containing 0.1M NaCl. Polyethylene glycol 6000 was added to obtain a final concentration of 3.5% (w/v) and, after mixing on a roller for one hour at room temperature, centrifuged at 2,700xg for 1 hour at 20°C. The supernatant was removed to a fresh tube and solid polyethylene glycol 6000 added to obtain a final concentration of 12% (w/v). The latter was mixed for one hour on a roller at room temperature and centrifuged at 2,750xg for 1 hour at 20°C. The supernatant was discarded and the pellet dissolved in phosphate buffered saline pH 7.5 to a volume equal to that of the undiluted harvested egg yolk.

3.1.3.3 Assessment

3.1.3.3.1 Triglyceride removal

Triglyceride (GPO-Trinder) working reagent was prepared by combining 4mL of Triglyceride Reagent A with 1mL Reagent B and warming to 37°C. The spectrophotometer was set at a wavelength of 540nm and the absorbance reading was set to zero with water as a reference. Then 1.0mL of working reagent was dispensed into disposable cuvettes and 10µl of distilled water, standard or sample added and incubated for 5 minutes at 37°C.

The absorbance of blanks, standards and samples at 540nm was recorded against water.

Assay principle (McGowan et al. 1983)

Hydrolysis of triglycerides is catalyzed by lipase to produce glycerol and free fatty acids. The glycerol generated is then phosphorylated by adenosine 5'- triphosphate in the presence of glycerol kinase. Oxidation of the glycerol 3-phosphate to produce hydrogen peroxide is catalyzed by L- α -glycerophosphate oxidase. An intense red chromogen is produced by the peroxidase catalyzed coupling of 4-aminoantipyrine and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate with hydrogen peroxide. The chromogen system facilitates a linear response to serum triglyceride concentrations.

3.1.3.3.2 IgY recoveries

This was determined by a specific fluoroimmunoassay for chicken immunoglobulin Y

described below. An antibody solid phase was prepared by covalently coupling 0.05mL of ovine anti-chicken IgY serum per gram of Sepharose, following the manufacturer's instructions. Chicken IgY was labelled with FITC. A stock solution of Sigma chicken IgY was prepared in saline (150mM NaCl) and the protein concentration determined by absorbance at 280nm with an extinction coefficient of 1.4 (1g/L solution, 1cm path length, Sigma product information).

IgY standards (doubling dilutions of a 1g/L solution) were prepared by appropriately diluting the stock solution in borate buffer (4.8g/L di-sodium tetraborate, pH 9, containing 1mL/L Triton X-100). To duplicate tubes containing 50 μ L of each standard was added 200 μ L (1.6 μ g IgY) of fluorescein-labelled IgY, followed by 100 μ L (10mg) of the anti-IgY solid-phase in borate buffer. The tubes were incubated for 30min at room temperature while mixing on a mechanical shaker. Volumes were made up to 1mL by addition of borate buffer and separation of the unbound fraction achieved by centrifugation for 5 minutes at 2750xg and 4°C followed by aspiration of the supernatant. Then 1.5mL of elution reagent (0.1M sodium hydroxide, containing 1mL/L Triton X-100) was added and mixed for 10min at room temperature on a mechanical shaker. After centrifugation for 5 minutes at 2750xg at 4°C the fluorescence intensities of the supernates were determined using a Perkin-Elmer LS-20 filter fluorimeter.

3.1.3.3.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were prepared between two glass plates which had been thoroughly cleaned using distilled water and acetone. The plates were separated using Teflon spacers with the base of the gel sealed using a rubber seal onto which the glass plates were pressed. Separating gel (Table 3.1) was poured between the plates and then covered by an overlay solution (acetone) to ensure even setting at the acrylamide/acetone interface. Once set, the overlay solution was washed off with distilled water and the stacking gel (Table 3.1) poured and a Teflon comb inserted at the top to form the wells. Once set, the comb was

removed and the sample wells and upper and lower buffer chambers filled with running buffer (Tris 25mM, glycine 0.192M, sodium dodecyl sulphate 0.1% w/v, pH 8.3) before use.

The samples were diluted at least 1:4 with non-reducing sample buffer containing 62.5mM Tris-HCl (pH6.8), 2% (w/v) SDS, 10% glycerol and 0.05% (w/v) bromophenol blue for non-reducing conditions. The molecular weights of unknown proteins were calculated by running the molecular weight standards, myosin (205kDa), β -galactosidase (116kDa), phosphorylase b (97kDa), fructose-6-phosphate kinase (84kDa), albumin (66kDa), glutamic Dehydrogenase (55kDa), ovalbumin (45kDa), and glyceraldehyde-3-phosphate dehydrogenase (36kDa) on the same gel and plotting a calibration curve of the R_f (distance moved by solute from origin divided by distance moved by solvent from origin) against molecular weight.

Gels were run at a constant voltage (150V) and visualised by staining with Coomassie brilliant blue G 0.05% (w/v) for 1 hour before being destained with water/acetic acid/methanol mixture 5:1:4 prior to drying.

3.1.3.4 Immunisation of hens with snake venoms

3.1.3.4.1 Immunogen preparation

Twenty mg of each of the three European viper venoms was weighed, dissolved in 2mL of saline and mixed together to give a total volume of 6mL at a concentration of 10mg/mL.

The stock venom mixture was stored at -20°C and from this stock the doses for each group in Table 3.2 were prepared.

3.1.3.4.2 Immunisation, serum sampling and egg collecting

This was carried out by MicroPharm Ltd. according to the schedule in Table 3.2 with three hens per dose group. Employing i.m. pectoral immunizations.

3.1.3.4.3 Yolk and serum preparation for titre assessment

	Separating Gel			Stacking Gel
	7.5%	10%	12%	
Water	4.85mL	3.85mL	3.15mL	6.1mL
1.5M Tris-HCl, pH 8.8	2.5mL	2.5mL	2.5mL	Not applicable(N/A)
0.5M Tris-HCl, pH 6.8	N/A	N/A	N/A	2.5mL
10%(w/v)SDS stock	100µL	100µL	100µL	100µL
Acrylamide/Bis (30 / 0.8% w/v)	2.5mL	3.5mL	4.2mL	1.3mL
10% ammonium persulphate	50µL	50µL	50µL	50µL
TEMED	5µL	5µL	5µL	10µL

Table 3.1 Constituents of SDS-PAGE gels

The white was separated from the yolk of every egg within a few days of laying and each yolk was then frozen in a 50mL centrifuge tube at -20°C until assessed (See section 3.1.3.1, p.83). For assessment, the yolks were thawed at room temperature and each yolk volume topped up with saline to a volume of 50mL and centrifuged at 2750xg for 20 minutes at 25°C and the supernatant collected. Yolk and serum samples for the three hens in each group were pooled and initially diluted 1:100.

3.1.3.4.4 Serum titre and egg yolk assessment by ELISA

Serum and egg yolk titres directed against European viper venoms, were determined by ELISA. Polystyrene 96-well micro-titre plates were coated with a solution of mixed venom (2mg/mL in sodium carbonate/bicarbonate buffer 0.1M, pH 9.6) by incubating 100µL per well for 2 hours at 37°C. Coated plates were washed three times with 300µL of ELISA washing buffer per well (NaCl 137mM, Na₂HPO₄ 8.1mM, KCl 2.68mM, NaH₂PO₄·2H₂O 1.28mM, Thimerosal 0.247mM, Tween 0.1%(v/v)), blocked with ELISA washing buffer for a further 2 hours at 37°C and finally washed to remove any unbound antigen. The plates were then incubated with 100µL of doubling dilutions of the immune serum diluted with ELISA washing buffer. A reference antiserum (from a pool of sera with an established titre) and a non-specific sheep serum were included on each plate as controls.

All dilution curves were performed in duplicate. Plates were incubated at 37°C for 1 hour, washed three times and then incubated with 100µL of donkey anti-chicken immunoglobulin (1:1000 dilution in ELISA washing buffer) to which had been conjugated horseradish peroxidase (HRP). Colour development was achieved at room temperature using 100µL of o-phenylenediamine dihydrochloride (OPD, 0.1mg/mL in citrate buffer, 0.07M, pH 5.0) with hydrogen peroxide (0.02%) and stopped after 15 minutes with 50µL of 3M sulphuric acid. The optical density (492nm) was read using a Titertek Multiscan PLUS (MKII) plate reader and the mean of the duplicates calculated.

Weeks	Actions	Venom dose		
		Low dose (mg/Hen)	Medium dose (mg/Hen)	High dose (mg/Hen)
0	Immunisation/Serum sampling/Egg collecting	0.1	0.1	0.1
4	Immunisation	0.25	0.25	0.25
6	Serum sampling /Egg collecting			
8	Immunisation	0.25	0.5	1.0
10	Serum sampling /Egg collecting			
12	Immunisation	0.25	0.5	1.0
14	Serum sampling /Egg collecting			
16	Immunisation	0.25	0.5	1.0
18	Serum sampling /Egg collecting			
20	Immunisation	0.25	0.5	1.0
22	Serum sampling/Egg collecting			

Table 3.2 Immunisation protocol for hens

Titres were determined at 50% of the background normal chicken serum corrected absorbance and values corrected for between assay variation using the reference antiserum.

3.1.3.5 Caprylic acid and ammonium sulphate precipitation. Replicate extractions

Five replicate extractions and purifications were carried out as previously described on yolk from eggs laid by the three hens on the low dose immunisation schedule (Table 3.2) at 22 weeks. Fifteen egg yolks were prepared as previously described and pooled and frozen at -20°C. The average yolk volume was 15mL. Replicate extractions were carried out using 5mL aliquots of the thawed pooled yolk and all results scaled up to 15mL.

3.1.3.6 Affinity purification of caprylic acid and ammonium sulphate precipitated IgY

A 1:1:1 mixture of *V.a.ammodytes*, *V.b.berus* and *V.a.aspis* venom was coupled to CNBR activated 4 Fast Flow Sepharose (5mg/g) following the manufacturer's instructions. The coupling efficiency for the venom:Sepharose conjugate was calculated as described elsewhere (See page 70, section 2.2.3.1) and was satisfactory.

50mL of a 17g/L solution of IgY prepared from eggs laid by three hens on the low dose immunisation schedule (Table 3.2) was recirculated overnight at 2mL/min. in the column described above. The bound material was then processed as described in section 2.2.3.2 except that the eluted affinity purified IgY was immediately neutralized with an equal volume of 1M trisodium citrate and then dialysed against a 20mM sodium chloride solution and finally freeze dried. From approximately 850mg of total protein loaded onto the affinity matrix, 8mg of specific IgY (1%) was collected and freeze dried.

3.1.3.6.1 Assessment

The replicate extractions were assessed for triglyceride concentrations, IgY recovery, purity by SDS - PAGE and activity by ELISA. The potency of the IgY was assessed before and after affinity purification by ED₅₀ as described in Chapter 4.

3.1.4 Results

3.1.4.1 Assessments

Fresh yolk was used throughout unless otherwise stated.

3.1.4.1.1 Triglyceride removal

Ninety nine percent or greater of the triglycerides were removed by all the Step 1 extraction procedures studied (Table 3.3) except for dilution, freezing and thawing which removed approximately ninety five per cent.

3.1.4.1.2 IgY recoveries

IgY recovered for Steps 1 and 2, respectively, were 163mg and 144mg for caprylic acid and ammonium sulphate, 162mg and 80mg for dextran sulphate and sodium sulphate, 151mg and 150mg for chloroform and PEG, 104mg and 28mg for dilution, freeze, thaw and sodium sulphate and 85mg and 87 mg for PEG (3.5% and 12%) using fresh yolk (Figure 3.1(a)).

The values of 163mg, 162mg, 151mg, 104mg and 85mg respectively for the different Step 1 lipoprotein extraction methods (Figure 3.1(a)) using fresh yolk decreased to 150mg, 126mg, 135mg, 37mg and 66mg when the same yolk source was frozen and thawed prior to extraction (Figure 3.1(b)).

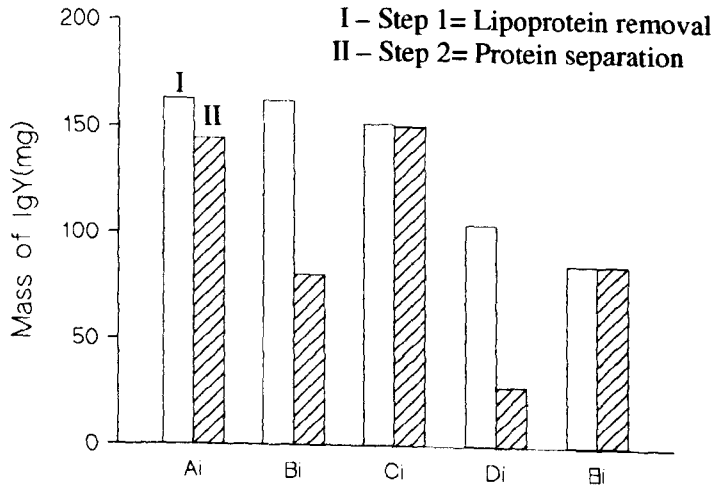
3.1.4.1.3 Purity by SDS-PAGE

Purification by the Step 1 plus Step 2 methods above (Figure 3.2(a) and (b)) did not result in IgY preparations of satisfactory purity. Thus the preparations contain proteins of less than 36kDa and up to 66kDa as well as IgY. However, IgY purification by caprylic acid and ammonium sulphate followed by two washes (Figure 3.3(a) and Figure 3.3(b)) resulted in an IgY preparation of satisfactory purity where most unwanted proteins have been removed.

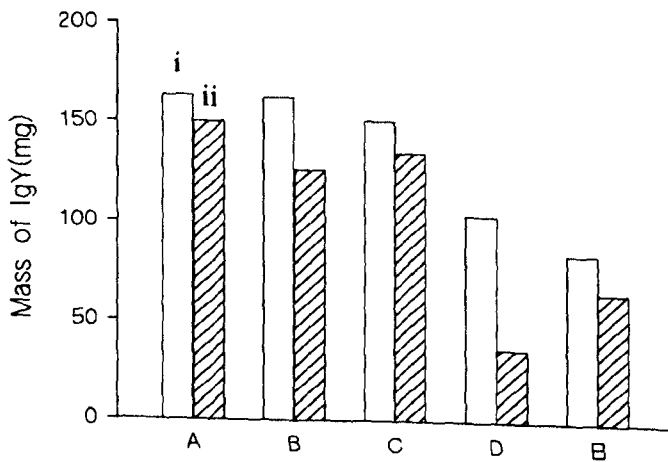
3.1.4.1.4 Assessment of serum and egg yolk titres by ELISA

Method	Triglyceride concentration in the product (mg/dL)	Volume from 5mL of yolk (mL)	Remaining triglycerides for a typical egg (15mL of yolk) (mg)	Percentage remaining triglycerides from total yolk
Caprylic acid	22	9.5	6.3	0.2
PEG	28	9	7.8	0.3
Chloroform	30	11	10	0.4
Dextran sulphate	44	27	36	1
Dilution, freezing and thawing	106	45	143	5
Total yolk not processed	18100	15	2715	100

Table 3.3 Summary of triglyceride assessments for Step 1 extraction procedures from Lion egg yolks

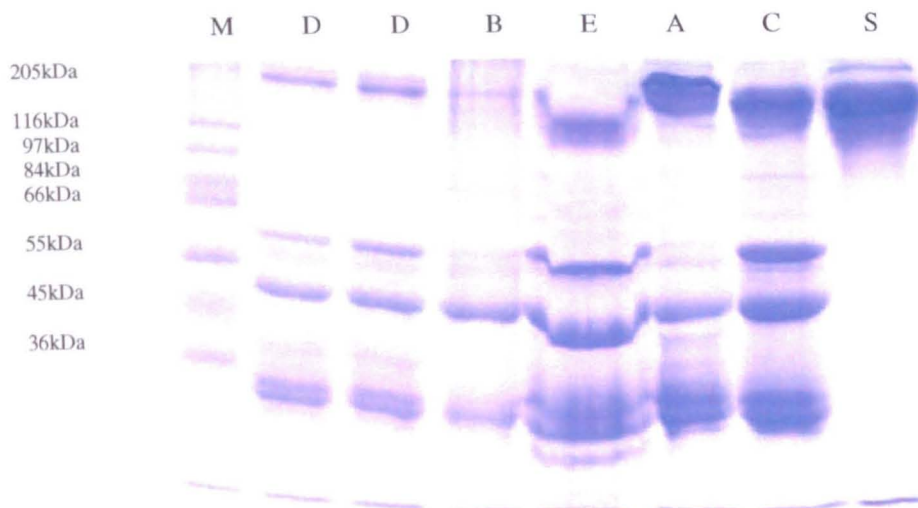


(a). IgY recovery from 15mL of fresh egg yolk after purification with caprylic acid and ammonium sulphate (Ai), dextran sulphate and sodium sulphate (Bi), chloroform and PEG (Ci), dilution, freezing and thawing and sodium sulphate (Di) and PEG (Ei).

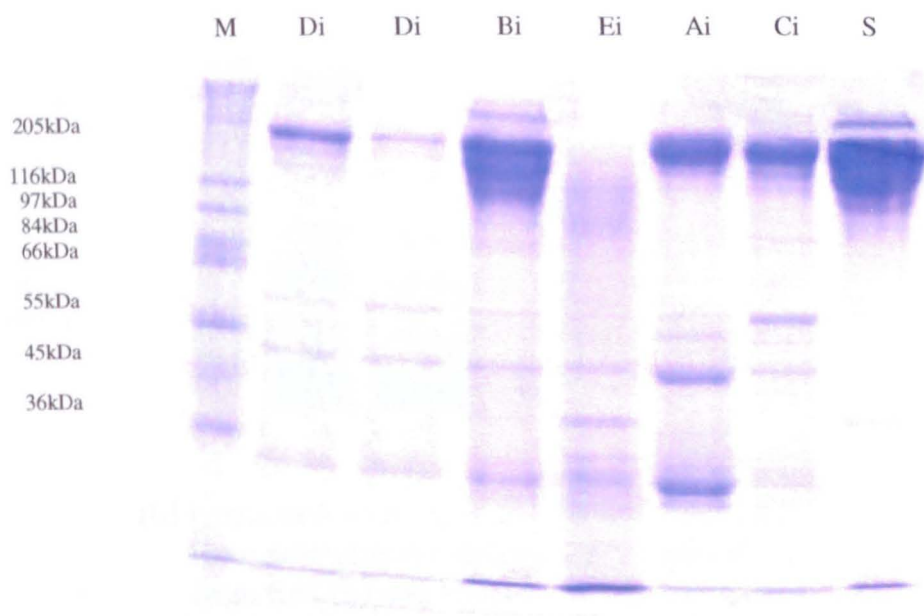


(b). IgY recovery from a typical fresh (i) and frozen (ii) egg yolk (15mL) after Step 1 lipoprotein extraction with caprylic acid(A), dextran sulphate (B), chloroform (C), dilution, freezing and thawing (D) and PEG (E).

Figure 3.1 Use of IgY immunoassay to monitor IgY purification procedures (a) and to assess the effect of yolk freezing on IgY recovery during lipoprotein extraction (b).

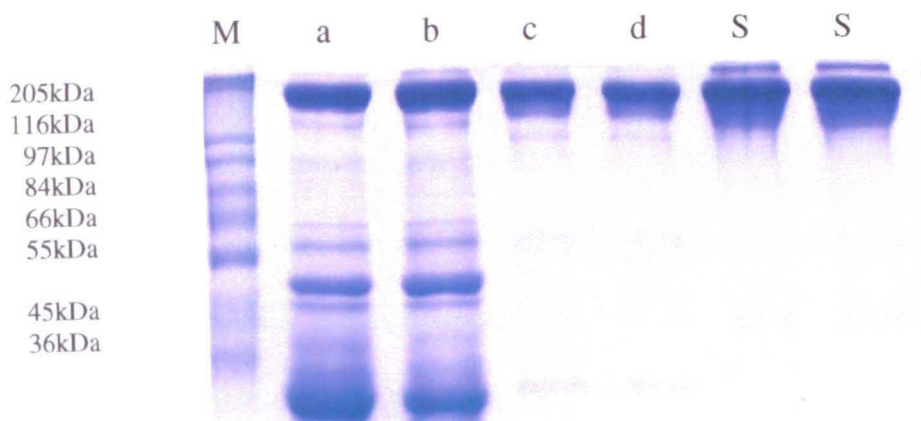


(a). Lipoprotein extraction by dilution, freezing and thawing (D), with dextran sulphate (B), PEG (E), caprylic acid (A), and chloroform (C). (M) molecular weight markers and (S) Sigma IgY.

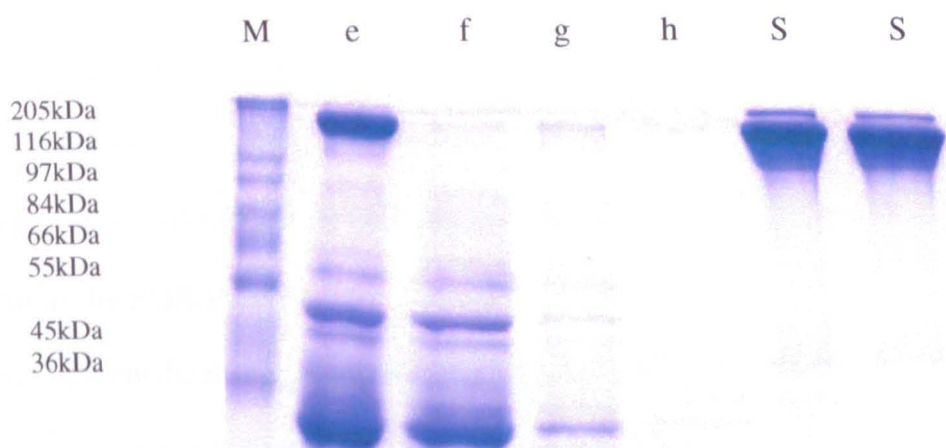


(b). IgY purification by dilution, freezing and thawing and sodium sulphate (Di), dextran sulphate and sodium sulphate (Bi), PEG (Ei), caprylic acid and ammonium sulphate (Ai) and chloroform and PEG (Ci).

Figure 3.2 Lipoprotein extraction (a) and IgY purification (b) as assessed by SDS-PAGE (7.5% (v/v)).



(a). IgY purification by caprylic acid and ammonium sulphate with two washes. Extraction with caprylic acid (a), precipitation with 1.75M ammonium sulphate (b), first wash (c) and second wash (d) with 1.75M ammonium sulphate. Molecular weight markers (M) and Sigma IgY(S).



(b) Extraction with caprylic acid (e), supernatant collected from ammonium sulphate precipitation (f), and supernatant from first (g) and second (h) washes.

Figure 3.3 IgY purification by caprylic acid and ammonium sulphate precipitation with two washes as assessed by SDS-PAGE (10%(v/v)).

Frozen and thawed egg yolk was used throughout unless otherwise stated.

Figure 3.4(a) and (b) show the time course of the immune response from pooled serum and egg yolk samples respectively from the low, medium and high dose groups. Taking into consideration the variability involved in ELISA, after 14 weeks the yolks have higher titres on average while after 22 weeks the sera appears to have slightly better titres. The hens responded better to immunisation with the low dose of venom than with the medium and high doses.

3.1.4.2 Assessment of reproducibility of IgY purification

Frozen and thawed egg yolk was used throughout unless otherwise stated.

3.1.4.2.1 Triglyceride removal

Mean removal of triglyceride from (Figure 3.5, Table 3.4) is $98.9\% \pm 0.33$ (mean \pm SD).

3.1.4.2.2 IgY recovery

Values of $86\text{mg} \pm 10\text{mg}$ (mean \pm SD) after triglyceride removal with caprylic acid, $72\text{mg} \pm 11\text{mg}$ (mean \pm SD) after precipitation with 1.75M ammonium sulphate and $68\text{mg} \pm 13\text{mg}$ (mean \pm SD) after first wash and $67\text{mg} \pm 12\text{mg}$ (mean \pm SD) after second wash.

3.1.4.2.3 Purity by SDS-PAGE

The replicate IgY purifications (Figure 3.6 (a)) resulted in IgY preparations of satisfactory purity where most unwanted proteins have been removed.

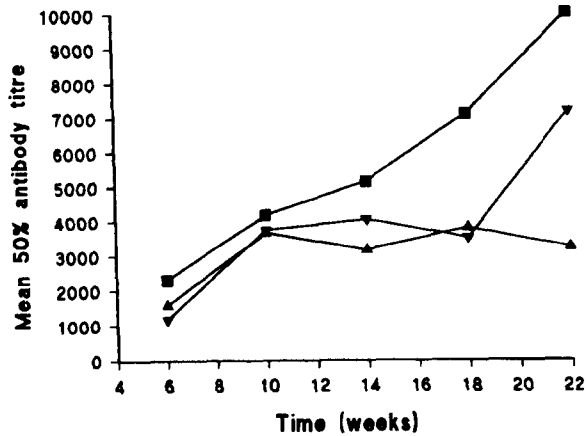
3.1.4.2.4 ELISA titre recovery

Values of 50% antibody titre of 876 ± 44 (mean \pm SD) after triglyceride removal with caprylic acid and 776 ± 63 (mean \pm SD) after second wash with 1.75M ammonium sulphate (Figure 3.6(b)).

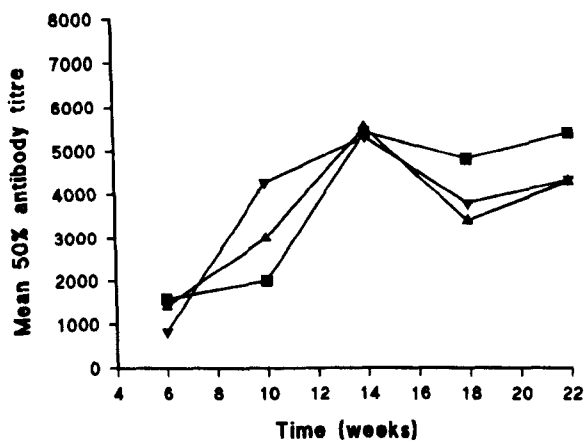
3.1.4.2.5 Potency of total and specific IgY

ED₅₀ result quoted with 95% confidence limits in parenthesis.

The total IgY did not protect the mice when the latter were tested with a mixture (1:1:1) of *V.a.ammodytes*, *V.b.berus* and *V.a.aspis* venoms and thus specific antibodies had to be affinity purified from the total IgY. Potency of specific antibodies was estimated at $113\mu\text{g}$



(a). ELISA titres for European viper antiserum samples pooled from each group(n=3) of hens immunised with low (■), medium (▲) and high (▼) mixed venom doses.

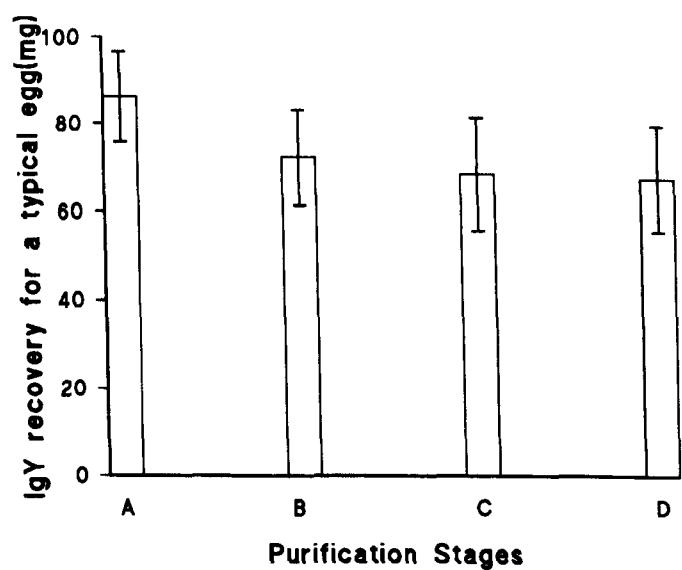


(b). ELISA titres for egg yolk samples pooled from each group(n=3) of hens immunised with low (■), medium (▲) and high (▼) mixed venom doses.

Figure 3.4 Serum (a) and egg yolk (b) titre assessments by ELISA over a 22 week period.

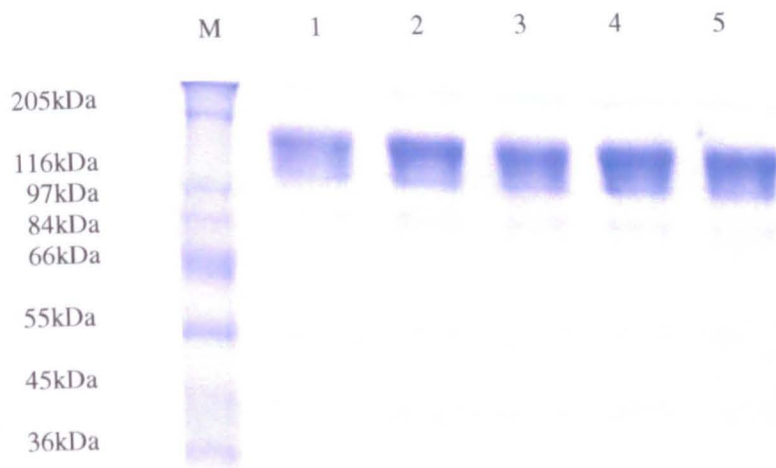
Sample Number	Triglyceride concentration in the product (mg/dL)	Volume From 5mL of yolk (mL)	Remaining triglycerides for a typical egg (15mL of yolk) (mg)	Percentage remaining triglycerides from total yolk
1	169	5.7	29	1
2	125	6.7	25	0.9
3	159	6.7	32	1
4	150	6.5	29	1
5	256	6	46	1.7
Total yolk not processed	18100	15	2715	100

Table 3.4 Triglyceride assessment for five replicate extractions with caprylic acid from viper venom immunised chicken yolks.

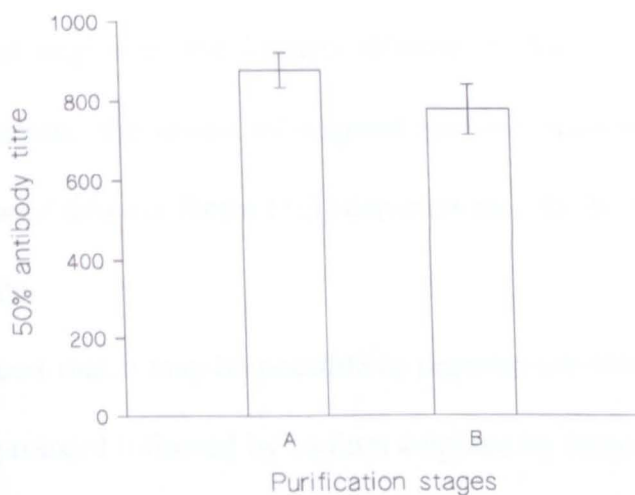


(a). IgY recovery from the extraction with caprylic acid(A), precipitation with 1.75M ammonium sulphate(B), first wash(C), and second wash(D) with 1.75M ammonium sulphate.

Figure 3.5 Summary table of remaining triglycerides and IgY recovery from total yolk after five replicate purifications with caprylic acid and ammonium sulphate.



(a). Five (1, 2, 3, 4, 5) replicate IgY purifications by caprylic acid and ammonium sulphate with two washes assessed by SDS-PAGE (7.5%(v/v)). Molecular weight markers(M).



(b). ELISA titre recovery of specific IgY from the extraction with caprylic acid (A) and after a second wash (B) with 1.75M ammonium sulphate.

Figure 3.6 SDS-PAGE and ELISA titre of replicate IgY purifications.

(96µg-130µg) of IgY per mouse when the latter were tested with the (1:1:1) venom mixture. Assuming that the capacity of the affinity matrix had not been exceeded, the recovery of 8mg of specific from 850mg of total suggests that approximately one percent of total IgY is specific to the venom constituents.

3.1.5 Discussion

Avian egg yolk contains at least 30% by weight of lipid with 67% of the lipid being triglycerides (Burley, 1971). Thus it was reasonable to monitor the effectiveness of the lipoprotein removal protocols studied here by measuring the disappearance of triglycerides. Dilution, freezing and thawing removed approximately 95% while at least ninety nine percent were removed by all the other protocols when using fresh yolk. Caprylic acid and ammonium sulphate and chloroform and PEG demonstrated the highest and comparable IgY recoveries. The dextran sulphate and sodium sulphate and PEG demonstrated lower recoveries and freeze and thaw and sodium sulphate demonstrated the lowest recovery.

It may be that the recovery of IgY and its dilution factor in the relevant lipoprotein removal protocol is affecting its subsequent precipitation by salt fractionation. Thus the lipoprotein removal step with the highest dilution factor (1:10), that is dilution, freeze and thaw demonstrates the lowest subsequent recovery with sodium sulphate, and caprylic acid with the lowest dilution factor (1:3) demonstrates the best subsequent recovery with ammonium sulphate.

It follows that it may be possible to improve recovery of IgY using the dilution, freeze and thaw protocol followed by sodium sulphate by attempting to reduce the dilution step during lipoprotein removal. The dilution, freeze and thaw method has the advantage of being inexpensive but fails to remove as much triglyceride as the other protocols studied. This therefore seems to lack promise.

It was necessary to study the effects of freezing yolks and the subsequent recovery of IgY because all the yolk from immunised hens with European Viper venoms at our disposal had

been stored frozen. These studies show that freezing affects IgY recoveries with all lipoprotein removal protocols but that lipoprotein removal with caprylic acid is affected the least. It is recommended, based on these results, not to store yolk frozen prior to IgY purification if possible. Also, it is necessary to study what is the effect on eggs or their yolks if they are kept at 4°C. and not frozen and for how long they can be stored in this way and still be useful.

Assessment by SDS-PAGE shows that a one step purification protocol post-lipoprotein removal is not enough and that in the case of caprylic acid and ammonium sulphate two subsequent washes with the latter (1.75M) were required.

The assessment of serum and egg yolk titres, in viper venom immunized-hens, by ELISA showed that these hens responded better to a low than a medium or high dose but that overall only approximately one percent of the total IgY was specific. This suggests that to produce an effective antivenom with the current immunisation protocol we would have to affinity purify the total IgY product because the latter was shown not to protect the mice in the relevant potency assessment. This adds complexity and cost to the manufacture of the antivenom.

Further work in the area of selection of animal species is indicated by these results.

Studies of reproducibility of purification were carried out using caprylic acid followed by ammonium sulphate (1.75M) precipitation and two further ammonium sulphate washes because at this stage in the work the latter purification protocol was proving the best candidate for frozen egg yolk mainly from the point of view of IgY recovery. The triglyceride concentrations remaining after lipoprotein removal averaged one percent compared with 0.2% for Lion eggs and the IgY recoveries were approximately two thirds of those obtained from Lion eggs. This difference may be due to the different yolk source, the effects of prolonged freezing or both; this requires further investigation.

Overall, caprylic acid and ammonium sulphate with two washes for lipoprotein removal

and IgY purification respectively results in good IgY recovery of satisfactory purity and ELISA titre. The use of caprylic acid for lipoprotein removal has not been promoted in the scientific literature but these studies have shown it to be a good protocol both from the point of view of triglyceride removal and IgY recovery. One practical disadvantage of caprylic acid is that it produces an infranatant aqueous layer, with the lipid mass resting on its surface, which makes the removal of the liquid difficult. This requires further investigation if it is decided to scale up the protocol for large scale processing of egg yolk.

3.2 Enzymatic cleavage of IgY from chicken egg yolk with proteolytic enzymes

3.2.1 Background

Porter (1959) used proteolytic enzymes to elucidate antibody structure and showed that rabbit IgG antibodies could be split by the proteolytic enzyme papain into three large fragments. He concluded that two of the fragments were identical and retained univalent binding capacity (Fab fragments), whereas the third could be crystallised (Fc fragment). Today, enzyme cleaved antibodies, mainly of equine origin are used widely throughout the world for the treatment of, for example, victims of snake and arthropod envenoming (DosSantos et al. 1989; Smith et al. 1992; Jones and Landon, 2002).

The yolk of eggs laid by immunised chickens has been recognised as an excellent source of polyclonal antibodies by Polson (1980) and Jensenius (1981), with potential for diagnostic and therapeutic applications.

Akita and Nakai (1993) describe methods for the production of Fab and Fab' fragments from chicken egg yolk IgY by papain and pepsin digestion respectively. The latter was achieved by first removing the lipoproteins using water dilution and then carrying out successive precipitations with ammonium and sodium sulphate before digestion.

The aim of the present work is to produce chicken egg yolk IgY fragments of acceptable purity for therapeutic application by directly digesting the extract resulting from lipoprotein

removal using caprylic acid and thus by-passing the need for a salt fractionation step post lipoprotein removal and predigestion.

3.2.2 Materials

Chicken immunoglobulin (IgY) was obtained from Sigma. Egg yolk from hens immunised with a mixture of European viper venoms (*V.berus*, *V.ammodytes* and *V.aspis*) was obtained from MicroPharm Ltd; centrifuge tubes from Greiner Labortechnik; caprylic(N-octanoic) acid and papain were purchased from BDH; dialysis membrane was obtained from Medicell; and all other chemicals were purchased from Sigma.

3.2.3 Methods

3.2.3.1 Proteolytic enzyme digestion of a commercial preparation of IgY

Unless stated otherwise all assessments were carried out with SDS-PAGE (7.5%(v/v)).

3.2.3.1.1 Papain digestion

Papain 16% (w/w) was added to IgY (10g/L) prepared in 100mM phosphate buffer, pH 7.0, containing 3mM EDTA and 10mM cysteine hydrochloride. During incubation at 37°C samples were removed at set time intervals and the reaction was terminated by adding iodoacetamide to each sample to a final concentration of 30mM.

3.2.3.1.2 Trypsin digestion

Trypsin 2% (w/w) was added to IgY (10g/L) prepared in 100mM Tris buffer pH 8. During incubation at 37°C, samples were removed at set time intervals and the reaction was terminated by adding phenylmethylsulphonyl fluoride to each sample to a final concentration of 100mM.

3.2.3.1.3 Pepsin digestion

Pepsin 2% (w/w) was added to IgY (10g/L) prepared in 50mM sodium acetate buffer at pH 4.2. During incubation at 37°C samples were removed at set time intervals and the reaction was terminated by adjusting the pH of each sample to around 6.

A pool of frozen and thawed egg yolk was used throughout unless otherwise stated.

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added to a concentration of 1.75M. After stirring at 4°C for one hour followed by centrifugation for 10 minutes at 2750xg and 25°C, the supernatant was discarded and the pellet washed by resuspending in 1.75M ammonium sulphate to a volume equal to that of the undiluted harvested egg yolk and recentrifuged. A second wash with 1.75M ammonium sulphate was carried out. The washed pellet was dissolved in 50mM sodium acetate buffer at pH 4.2. and made up to an estimated concentration of 10g/L by comparison with the zero sample in figure 3.7(b) it can be seen that the IgY is affected by the ingredients of the papain digestion mixture. Thus degradation products of around 116 KDa can be observed before the enzyme is added.

3.2.4 Results

3.2.4.1 Proteolytic enzyme digestion of a commercial preparation of IgY.

(10mL) was dialysed against 10L of 50mM sodium acetate buffer, pH 4.2 at 4°C over three days with a 1x10L buffer change every twenty four hours to remove the ammonium fragments of molecular weight around 55 KDa are produced together with fragments smaller than 45 KDa. The intensity of the stained band corresponding to the 55kDa

The IgY prepared above was warmed to 37°C in an incubator and 2%(w/w) pepsin added. During incubation at 37°C samples were removed at set time intervals and the reaction was terminated by adjusting the pH to around 6.

3.2.4.1.1 Papain (Figure 3.7.(a))

3.2.4.1.2 Trypsin (Figure 3.7(b))

Fab fragments of molecular weight around 55 KDa are produced after one hour of digestion together with other randomly sized fragments spread across the molecular weight range described by the markers (116 KDa to 36KDa). This pattern persists for four hours. After 24 hours all fragments are digested except for a broad band of weakly stained material corresponding to a molecular weight of smaller than 55 KDa.

After 48 hours the latter had faded further.

3.2.4.1.3 Pepsin (Figure 3.8(a))

After one hour digestion with pepsin, IgY starting material and fragments of around 116 KDa can be observed together with Fab fragments of molecular weight around 55KDa. During a four hour period the stained band corresponding to IgY and the stained band corresponding to the 116 KDa fragment gradually decreases while the stained band corresponding to 55 KDa increases in intensity. After 24 hours the IgY band and the 116 KDa band have disappeared and there is only one remaining band corresponding to 55 KDa. The latter persists for 48 hours.

sulphate precipitation

Thirty millilitres of acetate buffer (0.06M, pH4) was added to 15mL of egg yolk and the pH

adjusted to 4.8 by dropwise addition of 0.2M NaOH. Then 304 μ l of caprylic acid per mL of yolk was added dropwise with vigorous mixing which was continued for one hour followed by centrifugation at 2750xg for one hour at 20°C. This produced an infranatant aqueous layer, which will be referred to as the caprylic acid extract, with the lipid mass resting on its surface. Centrifugation was carried out in clear plastic tubes and a needle used to pierce the latter and withdraw the aqueous layer leaving the lipoprotein mass behind. The infranatant was filtered through a glass fibre filter to remove egg yolk sac debris, its pH adjusted to 7.5 with 1M Tris buffer pH 8 and solid ammonium sulphate

3.2.3.2 Pepsin digestion of IgY prepared by caprylic acid followed by ammonium sulphate precipitation

Thirty millilitres of acetate buffer (0.06M, pH4) was added to 15mL of egg yolk and the pH adjusted to 4.8 by dropwise addition of 0.2M NaOH. Then 304 μ l of caprylic acid per mL of yolk was added dropwise with vigorous mixing which was continued for one hour followed by centrifugation at 2750xg for one hour at 20°C. This produced an infranatant aqueous layer, which will be referred to as the caprylic acid extract, with the lipid mass resting on its surface. Centrifugation was carried out in clear plastic tubes and a needle used to pierce the latter and withdraw the aqueous layer leaving the lipoprotein mass behind. The infranatant was filtered through a glass fibre filter to remove egg yolk sac debris, its pH adjusted to 7.5 with 1M Tris buffer pH 8 and solid ammonium sulphate added to a concentration of 1.75M. After stirring at 4°C for one hour followed by centrifugation for 10 minutes at 2750xg and 25°C, the supernatant was discarded and the pellet washed by resuspending in 1.75M ammonium sulphate to a volume equal to that of the undiluted harvested egg yolk and recentrifuged. A second wash with 1.75M ammonium sulphate was carried out. The washed pellet was dissolved in 50mM sodium acetate buffer at pH 4.2. and made up to an estimated concentration of 10g/L by reconstituting the pellet in approximately 10mL of acetate buffer. This was then confirmed by absorbance at 280nm with an extinction coefficient of 1.4 as 9.7g/L. The IgY solution (10mL) was dialysed against 10L of 50mM sodium acetate buffer, pH 4.2 at 4°C over three days with a 1x10L buffer change every twenty four hours to remove the ammonium sulphate.

The IgY prepared above was warmed to 37°C in an incubator and 2%(w/w) pepsin added. During incubation at 37°C samples were removed at set time intervals and the reaction was terminated by adjusting the pH to around 6.

3.2.3.3 The effect of pH on the pepsin digestion of caprylic acid extract

The caprylic acid extract was prepared as previously described (See Section 3.1.3.2.1 p.84) and a rough estimate of the protein concentration of the solution (38g/L) was determined by absorbance at 280nm with an extinction coefficient of 1.4. The extract was aliquoted into four x 5mL samples and the pH of each sample was adjusted to 3, 3.5, 4 and 4.5 respectively with 0.1M HCl and then warmed to 37°C in an incubator. Next, 2% (w/w) pepsin was added to each sample and during incubation at 37°C, samples were removed at set time intervals and the reaction was terminated by adjusting the pH to 6.

3.2.3.4 Comparison of recoveries of IgY fragments prepared with and without ammonium sulphate precipitation

3.2.3.4.1 Pepsin digestion of IgY prepared by ammonium sulphate precipitation

IgY fragments were prepared as previously described with the difference that after the second wash with 1.75M ammonium sulphate the pellet was dissolved in 50mM sodium acetate buffer, pH 4.2 and made up to a volume equal to that of the undiluted harvested egg.

Also the reaction with pepsin was carried out at 37°C over 24 hours and was terminated by adjusting the pH to 6 with 0.1M NaOH.

The resulting IgY fragments were dialysed in PBS pH 7.5 at 4°C to remove unwanted digested protein material over three days with a 1x10L buffer change every 24 hours. The protein concentration of the solution before and after dialysis was determined by absorbance at 280nm with an extinction coefficient of 1.4.

3.2.3.4.2 Pepsin digestion of IgY prepared without ammonium sulphate precipitation

The caprylic acid extract was prepared as previously described and its pH adjusted to 4.5 with 0.1M HCl and warmed to 37°C in an incubator. Then 2% (w/w) pepsin was added and the reaction solution incubated at 37°C over 24 hours. The reaction was terminated by adjusting the pH to 6 with 0.1M NaOH. The resulting IgY fragments were dialysed and assessed for protein concentration as described previously.

3.2.4 Results

3.2.4.1 Proteolytic enzyme digestion of a commercial preparation of IgY.

3.2.4.1.1 Papain (Figure 3.7.(a))

By comparison with the zero sample in figure 3.7(b) it can be seen that the IgY is affected by the ingredients of the papain digestion mixture. Thus degradation products of around 116 KDa can be observed before the enzyme is added.

No IgY starting material can be observed after one hour of digestion, by which time Fab fragments of molecular weight around 55 KDa are produced together with fragments smaller than 45 KDa. The intensity of the stained band corresponding to the 55kDa fragment increases during 48 hours and the intensity of the stained band corresponding to the latter decreases during the same time period but never disappears completely.

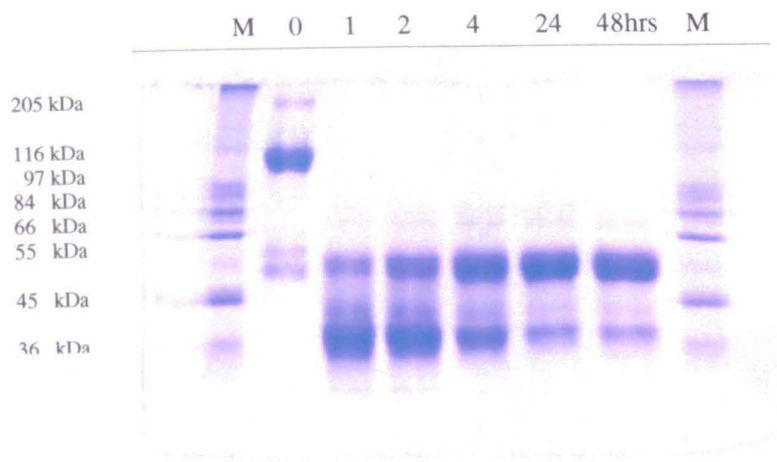
3.2.4.1.2 Trypsin (Figure 3.7(b))

Fab fragments of molecular weight around 55 KDa are produced after one hour of digestion together with other randomly sized fragments spread across the molecular weight range described by the markers (116 KDa to 36KDa). This pattern persists for four hours. After 24 hours all fragments are digested except for a broad band of weakly stained material corresponding to a molecular weight of smaller than 55 KDa.

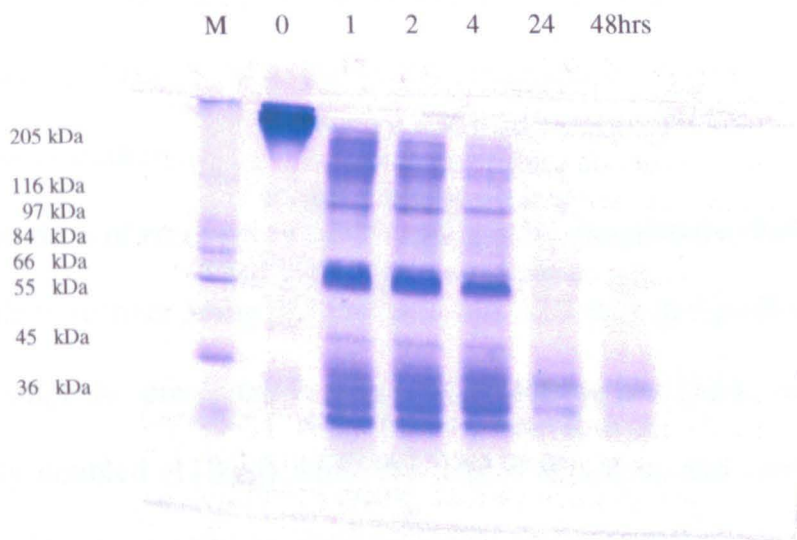
After 48 hours the latter had faded further.

3.2.4.1.3 Pepsin (Figure 3.8(a))

After one hour digestion with pepsin, IgY starting material and fragments of around 116 KDa can be observed together with Fab fragments of molecular weight around 55KDa. During a four hour period the stained band corresponding to IgY and the stained band corresponding to the 116 KDa fragment gradually decreases while the stained band corresponding to 55 KDa increases in intensity. After 24 hours the IgY band and the 116 KDa band have disappeared and there is only one remaining band corresponding to 55 KDa. The latter persists for 48 hours.



(a). Papain digestion



(b). Trypsin digestion

Figure 3.7 Proteolytic enzyme digestion of a commercial preparation of IgY

3.2.4.2 Pepsin digestion of IgY prepared by caprylic acid followed by ammonium sulphate precipitation (Figure 3.8(b))

Description the same as for figure 3.8(a).

3.2.4.3 The effect of pH on the pepsin digestion of caprylic acid extract

After one hour of digestion at pH 3 (Figure 3.9(a)) there is a faint band corresponding to 55 KDa fragments. This gets progressively fainter with time and after 48 hours has virtually disappeared. After one hour digestion at pH 3.5 (Figure 3.9(b)) a more discernible band in the same molecular weight range as the latter can be observed with a dispersed area of staining below it. In the next four hours the staining of the band corresponding to 55 KDa and the disperse area of staining below it gets progressively fainter until after 24 hours when it has virtually disappeared.

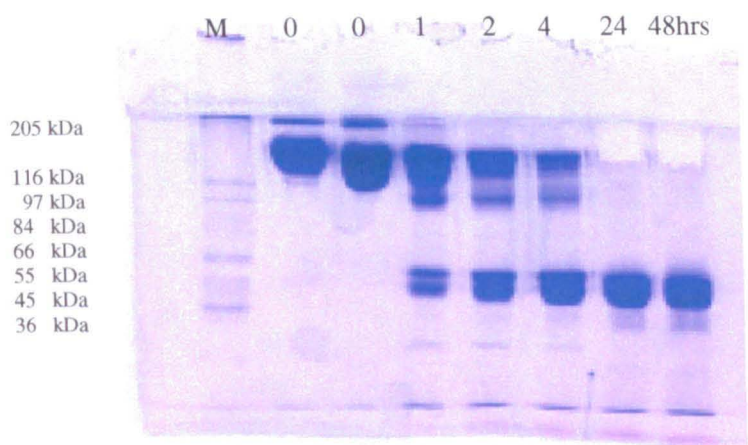
For up to four hours digestion at pH 4 (Figure 3.9(c)) results are similar to those obtained at pH3.5. However after 48hours the 55 KDa fragments persist albeit the staining of the band is weak. At pH 4.5 (Figure 3.9(d)) the 55 KDa fragment still persists after 48 hours with no significant loss of staining.

3.2.4.4 Comparison of recovery of IgY fragments by absorbance (280nm)

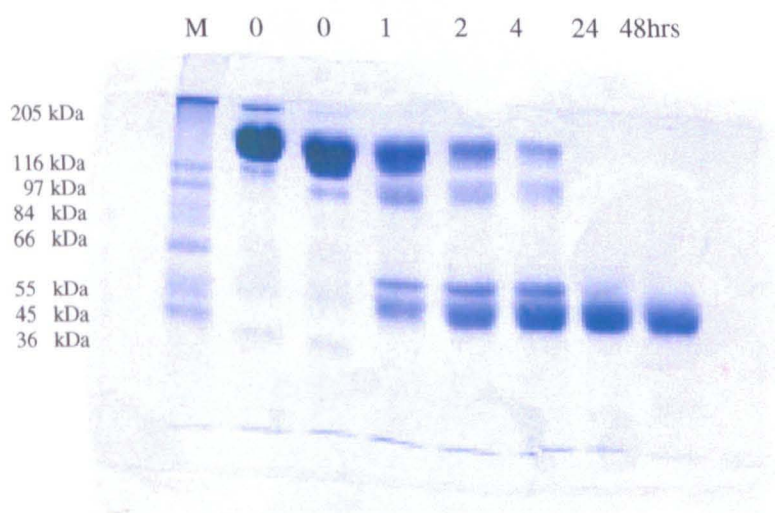
It was possible to recover 56mg of IgY fragments after pepsin digestion of IgY prepared by ammonium sulphate precipitation from a typical egg (15mL yolk). This recovery approximately doubled (110mg) when the pepsin digestion was carried out directly from the caprylic acid extract without carrying out the salt precipitation.

3.2.5 Discussion

Akita and Nakai (1993) determined the optimal pH conditions for IgY peptic digestion to be pH 4.2 and the enzyme: protein ratio 1:50 with a digestion time of 9 hrs. I adopted the recommended pH and enzyme:protein ratio in this study but felt it was important to carry out a timed digestion study as it was not possible at present to estimate the potency of the pepsin that was available, also Akita and Nakai (1993) did not give any potency data about the pepsin they had used. The results of the study shows that a 24 hr digestion time was



(a) Digestion of a commercial preparation of IgY

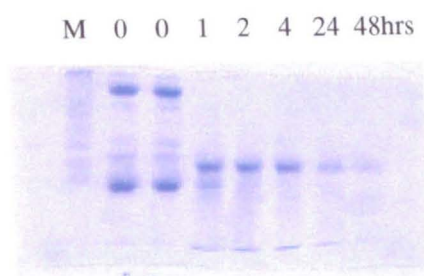


(b) Digestion of IgY prepared by ammonium sulphate precipitation

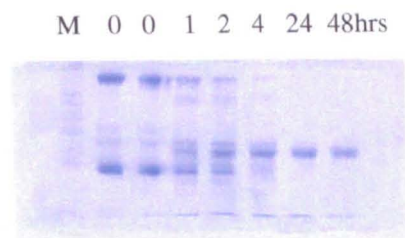
Figure 3.8 Pepsin digestion of IgY



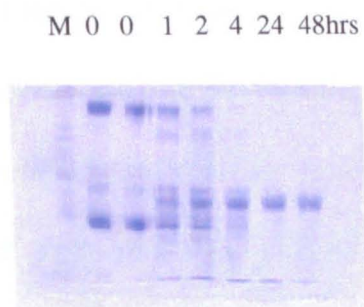
(a) pH 3



(b) pH 3.5



(c) pH 4.0



(d) pH 4.5

Figure 3.9 The effect of pH on the pepsin digestion of caprylic acid extract

necessary compared to the 9 hr recommendation by Akita and Nakai (1993) and thus feel that in view of this result the decision to carry out a timed experiment was justified.

Although other investigators (Jones and Landon, 2002) have shown that pepsin can completely digest serum proteins and produce $F(ab')_2$ from IgG nobody has shown until now that pepsin can completely digest egg proteins and produce Fab from IgY. As a result I feel it was fully justified to have investigated the time of digestion which was determined as 24 hours and pH which was determined at pH 4.5 conditions required for the digestion of a caprylic acid extract to produce Fab instead of relying on the previously optimised conditions for the digestion of IgY obtained from an ammonium sulphate precipitation of the caprylic acid extract.

All the enzymes studied produce Fab fragments when digesting IgY. This was also found by Akita and Nakai (1993), who studied pepsin and papain digestion of IgY. We can add the results of trypsin digestion to these findings.

Of the enzymes studied, only pepsin digests Fc fragments. The production of other fragments as well as Fab following trypsin and papain digestion of IgY means that further methods would need to be developed to remove them, thus adding to the cost and potentially increasing the loss of Fab in the process. Also, we have shown that pepsin can digest other proteins in egg yolk as well as the Fc component of the IgY, thereby doing away with the need to salt precipitate IgY so as to remove unwanted proteins prior to digestion. Pepsin digestion of IgY prepared without ammonium sulphate results in approximately twice the yield of Fab as when these are prepared from caprylic acid followed by ammonium sulphate precipitated IgY.

I have not shown that the activity of the fragments is conserved after digestion as this has been well documented by Jones and Landon (2002).

Although egg yolk has been recognized as a convenient and inexpensive source of antibodies it has not found widespread use as one might expect. This may be attributed in

part to difficulties in isolating IgY from yolk and to the potential for increased risks of allergenicity that may be associated with egg proteins. The protective effect of specific chicken antibodies should be conferred by either the whole IgY or the antigen binding fragment. Consequently for the people who may be allergic to egg protein attempts should be made to reduce or eliminate allergenicity. It is known that the Fc fragment is the most antigenic portion of the immunoglobulin molecule (Porter, 1959). Thus cleavage of the Fc fragment may be beneficial since it will reduce the number of allergenic sites on the IgY molecule without adversely affecting its function.

CHAPTER 4: THE PREPARATION AND EVALUATION OF A SPECIFIC OVINE ANTIVENOM AGAINST *V.LATASTEI* VENOM

4.1 Background

Gonzalez (1991) has treated successfully many victims of envenoming by *V.latastei* with antivenoms manufactured by Pasteur (IPSER Europe), by Behringwerke or Zagreb. These are all equine based and contain specific antibodies directed against *V. aspis*, *V.berus* and *V.ammodytes* venoms for the Pasteur product and, in addition, *V.lebetina* and *V.xanthina* venoms for the Behringwerke product. The Zagreb product is raised against only *V.ammodytes* venom but is described as being useful for all European snakes from the *Vipera* family. The studies of Detrait et al (1983) showed that *V. latastei gaditana* venom in mice may be neutralised by *V.berus*, *V.aspis* and *V.ammodytes* antisera which supports the clinical observations that a specific *V. latastei* antivenom is not necessary to treat cases of *V.latastei* envenomation.

A Spanish group (Pérez et al.1999), has welcomed a new antivenom, ViperfavTM (Institut Pasteur-Mérieux, France), as a replacement for IPSER Europe (Pasteur Mérieux, France). Thus whilst it contains the same specific antibodies as the latter (viz directed against *V.aspis*, *V.ammodytes* and *V.berus* venoms), it has been purified more extensively to obtain a product that can be given safely by the intravenous route, whilst the latter could only be used intra muscularly under its product licensing (though often given i.v. by clinicians). Nonetheless, Pérez and his colleagues (1999) argue that although ViperfavTM may solve the problem of anaphylactoid reactions in patients treated for envenomation by *V. latastei*, it does not contain antibodies directed specifically against *V. latastei* venom. They conclude that , since *V.latastei* bites are potentially serious and since most cases of snakebite in Spain are from these snakes, there may be justification for having specific antibodies against *V.latastei* venom in the Pasteur product.

Another argument for producing a specific *V.latastei* antivenom for the Iberian peninsula is presented by Cheng and Winkel (2001). They argue that there is a global loss of

momentum in antivenom research, development and financing. Although neglected, snake bite is eminently treatable and with this in mind we have been encouraged to manufacture a geographically relevant antivenom for Spain in collaboration with Dr Delfin Gonzalez, Spain's leading authority on snakebite. Then it is intended to help transfer the appropriate technology to enable Spain become self-sufficient in antivenom production.

The aim of the work was to prepare and evaluate a specific ovine F(ab')₂ antivenom for *V. latastei*. Sheep were immunised and their antisera pooled and used to produce antivenom by means of 'enhanced pepsin digestion' a method recently developed in our laboratories (Jones and Landon, 2002). We also extended this concept directly to whole blood since this could further simplify the process and increase yields. The potential disadvantages, such as a requirement for more pepsin than that used for serum digestion, larger processing volumes owing to initial dilution of the blood and decreased purity of the finished product, were also addressed.

4.2 Materials

V. latastei venom was kindly donated by Dr Delfin Gonzalez . Serum and blood from sheep immunised with *V. latastei* venom were obtained from MicroPharm Ltd and venoms of *V. ammodytes*, *V. aspis* and *V. berus* from Latoxan. CNBR activated 4 Fast Flow Sepharose and Q Sepharose fast flow were from Amersham Pharmacia Biotech. The chromatography system (Econo system) was obtained from Biorad. Lithium heparin tubes were purchased from Fisher Scientific. All other chemicals were purchased from Sigma.

4.3 Methods

4.3.1 Assessment of *V. latastei* venom and comparison with other European

Vipera venoms

4.3.1.1 Physical assessment

The venom samples were assessed by 12% SDS-PAGE as described elsewhere (See section 3.1.3.3.3, p.87).

4.3.1.2 Venom lethality

Venom lethality was tested by MicroPharm Ltd. using the median lethal dose (LD₅₀) method.

Principle (Klaassen and Eaton, 1985)

The LD₅₀ is defined as the dosage of venom required to produce 50% death in a population of outbred white mice. A range of venom doses (five in this case) containing increasing amounts of venom is tested such that all mice survive at the lowest dose group, die in the highest dose group and have some survivors in the remaining three groups.

Method

Where possible all solutions were sterile filtered (0.2µm) prior to use. The LD₅₀ of the venom (the amount that causes the death of 50% of the animals) was assessed by i.v. injection of various doses of venom in 0.2mL of physiological saline into the tail vein of 18-20g male out-bred white mice. Animals were placed under a heat lamp to dilate the tail vein and, thereby, aid injection. Five mice were used for each venom dose and the LD₅₀ was calculated from the number of deaths within 24hr by probit analysis. Animals were regularly checked during the 24hr period and dead animals removed. Any animals in severe distress and likely to die before the full 24hr period were humanely killed according to Home Office guidelines.

4.3.2 Production and assessment of antisera

4.3.2.1 Antisera production

The blood and serum used in the digestion protocols described below were obtained from sheep immunized with *V. latastei* venom every 28 days employing a dose regimen of 0.5, 1, 2, 4, 4, 4, 4, 4, 4, 4, 4mg over a forty-six week period. The concentration of specific antibodies in these sheep were assessed and the results discussed in Chapter 2.

Antisera were precipitated and washed (x2) with an 18% (w/v) final concentration of sodium sulphate to remove serum albumin before assessing its potency.

The concentration of IgG was estimated at 23g/L by absorbance at 280nm.

4.3.2.2 Antisera assessment

4.3.2.2.1 Potency of antisera

The effectiveness of the antisera was assessed by MicroPharm Ltd., using the effective dose (ED₅₀) test (which may also be termed the median potency dose) in outbred mice.

Principle (Klaassen and Eaton, 1985)

The ED₅₀ is defined as the dosage of antivenom required to protect or neutralise 50% of a population of treated animals against a multiple of the LD₅₀ of the venom. In all studies in this thesis, 2x LD₅₀ was used and the venom and antibody was pre-mixed and incubated for 30 min. prior to injection. A range of dose groups (five in this case) containing increasing amounts of antisera was studied such that all mice survive at the highest antisera dose group, die in the lowest dose group while there are some survivors in the remaining three groups.

Method

To assess antivenom potency, various amounts of antivenom were mixed with two times the LD₅₀ of venom; the mixture was then incubated at 37°C for 30 min and injected intravenously into mice. Five mice were used at each venom dose the ED₅₀ or median effective dose of the antivenom was calculated from the number of deaths within 24 hr after injection of the venom/antivenom mixture using probit analysis.

4.3.2.2.2 Binding of antisera to venom from other European vipers

4.3.2.2.2.1 Preparation of venom:Sepharose solid phase

V.l.latastei, *V.b.berus*, *V.a.ammodytes*, and *V.a.aspis* venom were coupled to CNBR activated 4 Fast Flow Sepharose (5mg/g) following the manufacturer's instructions. The coupling efficiency for each venom:Sepharose conjugate was calculated as described elsewhere.

4.3.2.2.2.2 Binding studies

All SSAP procedures were performed as previously described in Chapter 2.

V.latastei antisera were assessed on a *V.latastei*:Sepharose matrix and on similar matrices conjugated with *V.a.ammodytes*, *V.a.aspis*, and *V.b.berus* venoms.

4.3.3 Production of antivenom from antisera and assessment

4.3.3.1 Antivenom production by direct serum digestion

The method was adapted from Jones and Landon, 2002.

Pools of *V.latastei* antisera were diluted with 0.75 volumes of 0.116M HCl, to both adjust the pH of the serum to 3.5 and to dilute it. Pepsin was then added at a ratio of 2% (w/w) of total protein based on the assumption that the concentration of protein in serum is 100g/L. The digestion mixture was incubated overnight at 37°C, after which 200mM L-histidine base (HB) was added at a ratio of (volume of digestion mixture x 0.45 of HB) to adjust the pH to 6.0 and thereby stop the reaction. The digestion mixture was then centrifuged at 3,500 RPM for 45 minutes at 25°C and the supernatant collected and diafiltered (x10 volume) against 20mM HBS, pH 5.8 – 6.2). The diafiltered supernatant was then anion-exchanged using 40mL of Q Sepharose Fast Flow packed in a suitable column housing, with a 10cm bed height equilibrated with 200mL of HBS. The digested, diafiltered material was pumped down the column while monitoring A_{280nm} of the eluted material, followed by pumping fresh buffer (HBS) until the A_{280nm} returned to baseline. All the unbound material, corresponding to the purified $F(ab')_2$, was collected and stored at 2-8°C. Bound contaminants (acidic aggregates and pepsin) can be eluted to regenerate the column using a gradient with 20mM histidine buffer (pH 5.8-6.2) containing 1M NaCl. Flow rates of 10mL/min were used throughout.

4.3.3.2 Antivenom assessment

4.3.3.2.1 Purity

This was assessed by 7.5% SDS-PAGE as described elsewhere (See section 3.1.3.3.3, p87).

4.3.3.2.2 Potency

This was assessed by ED_{50} as described elsewhere (See section 4.3.2.2.1, p118).

The concentration of $F(ab')_2$ was estimated at 24g/L by absorbance at 280nm.

4.3.4 Assessment of Zagreb antivenom

4.3.4.1 Purity

This was assessed by 7.5% SDS-PAGE as described elsewhere.

4.3.4.2 Potency

This was assessed by ED_{50} as described elsewhere.

The concentration of equine immunoglobulin fragment ($F(ab')_2$) was estimated at 144g/L by absorbance at 280nm.

4.3.5 Investigation of production conditions required for making antivenom from blood

All blood used in these experiments was collected into lithium heparin tubes.

4.3.5.1 Dilution and acidification

A 2.5mL aliquot of ovine blood was diluted with an increasing volume 0.116M HCl to establish the amount of the latter required to adjust its pH to 2.5, 3, 3.5, 4 and 4.5 respectively.

4.3.5.2 pH conditions

The pH of 2.5mL x 5 aliquots of ovine blood were adjusted to 2.5, 3, 3.5, 4 and 4.5 respectively with 0.116M HCl and their final volume adjusted with water so that the original blood volume was diluted by a factor of 1 in 3. This was necessary because any lesser dilution resulted in the blood sample congealing during overnight incubation. The acidified blood was then warmed to 37°C in an incubator and 2% (w/w) pepsin added. During incubation at 37 °C, 0.5mL samples were removed at set time intervals of 0, 1, 2, 4, 24 and 48 hours. The reaction was terminated by adjusting the pH of each sample to 6 by adding 0.35mL of HB. The concentration of total protein in blood was assumed to be

200g/L.

4.3.5.3 Pepsin mass

The pH of 2.5mL x 2 aliquots of ovine blood were adjusted to 3.5 and the final volume adjusted with water so that the original blood volume was diluted by a factor of 1 in 3. The acidified blood was then warmed to 37°C in an incubator and 1% and 0.5% (w/w) pepsin was added to each respectively. During incubation at 37 °C, 0.5mL samples were removed at set time intervals of 0, 1, 2, 4, 24 and 48 hours. The reaction was terminated by adjusting the pH of each sample to around 6 by adding 0.35mL of HB.

4.3.5.4 Optimization of ion-exchange conditions

Twenty nine millilitres of blood containing antibodies to *V.latastei* venom were acidified with 48.6mL of 0.116M HCl. and a further 10mL of water was added to give a total volume of 87.6mL with the original blood being diluted by a factor of 1 in 3. The pH was adjusted to 3.5 and the acidified diluted blood then warmed to 37°C in an incubator; 2% (w/w) pepsin was added and the mixture incubated for a further 24 hours at 37°C. The digestion was terminated by adjusting the pH of the reaction mixture to around 6 by adding 60 mL of HB. After centrifugation at 2750xg for 45 minutes at 25°C, the supernatant was collected and diafiltered (x10 volume) against histidine buffer, pH 5.8 – 6.2 containing 20mM sodium chloride, employing a tangential-flow ultrafiltration unit with a 50 cm² 30,000 Da nominal molecular weight cut-off polyethersulphone membrane and a peristaltic pump capable of delivering a flow rate of 200-400 mL/min, at approximately 2.5 bar back pressure. The diafiltrate was aliquoted into four 20mL volumes. To three of these, sodium chloride was added to attain a concentration of 40, 80 and 150mM NaCl respectively.

Each of the above was anion-exchanged using 40mL of Q Sepharose Fast Flow as previously described with the proviso that the column bed was equilibrated with 200mL of the buffer containing the relevant sodium chloride concentration prior to the digested material being pumped down the column. The unbound material collected for each run was

scanned for absorption with a spectrophotometer over the range of 200 to 800nm and was also assessed for purity by 7.5% (v/v) SDS-PAGE.

4.3.5.5 Potency of antivenom produced from blood

This was assessed by ED₅₀ as described elsewhere.

The concentration of F(ab')₂ was estimated at 10g/L by absorbance (280nm).

4.3.5.6 Assessment of recovery of F(ab')₂ prepared from blood and serum

Blood (400mL) was obtained from a single sheep immunised as described in 4.3.2.1. and 200mL was processed by MicroPharm Ltd. to produce 134mL serum. The remaining 200mL was kept as blood. The ratio of serum to blood volume when comparing F(ab')₂ recoveries, was conserved at approximately 70% (v/v). Thus the recovery of F(ab')₂ from 30mL serum prepared as described in 4.3.3.1. was compared with that of F(ab')₂ prepared from 43mL of blood as follows.

The pH of 43mL of ovine blood was adjusted to 3.5 with 0.116M HCl and the final volume adjusted with water so that the original blood volume was diluted by a factor of 1 in 3. The acidified blood was then warmed to 37°C. in an incubator and 2% (w/w) pepsin was added. The reaction was terminated after 24 hours by adjusting its pH to around 6 by adding 79mL of HB and then centrifuging at 2750xg for 45 minutes at 25°C. The supernatant was collected and diafiltered (x10 volume) against histidine buffer, pH 5.8 – 6.2 containing 20mM sodium chloride employing a tangential-flow ultrafiltration unit with a 50 cm² 30,000 Da nominal molecular weight cut-off polyethersulphone membrane and a peristaltic pump capable of delivering a flow rate of 200-400 mL/min, at approximately 2.5 bar back pressure. The diafiltrate was aliquoted into two 20mL volumes. To the second volume, sodium chloride was added to attain a concentration of 40mM NaCl.

Each of the above was anion-exchanged as described previously. The unbound material collected for each run was assessed for purity by 7.5% (v/v) SDS-PAGE and for total protein by absorption at 280nm.

F(ab')₂ recoveries from the two methods were estimated by comparing the total protein at 280nm.

4.4 Results

All LD₅₀ and ED₅₀ results are quoted with 95% confidence limits in parenthesis (Table 4.1).

4.4.1 Assessment of *V.latastei* venom and comparison with other European *Vipera* venoms

4.4.1.1 Physical assessment

All the venoms exhibit protein bands that correspond to molecular weights from greater than 66kDa to smaller than 24kDa (Figure 4.1). Protein bands corresponding to approximate molecular weights 30 and 57 kDa can be found in all venoms. *V.berus* and *V.aspis* venoms have a protein band in common of approximately 40kDa. *V.berus* venom has a protein band not shared with other venoms of approximately 24kDa. *V.ammodytes* venom has an approximate 66kDa protein band not shared with other venoms and *V.aspis* venom has a protein band that it does not share with other venoms of approximately 62kDa.

4.4.1.2 Venom lethality

LD₅₀ for *V.latastei* venom was 17.8µg (13µg-22µg) per mouse. LD₅₀ for a 1:1:1 mixture of *V.a.ammodytes*, *V.b.berus*, and *V.a.aspis* venom was 6.7µg (6.3µg-7.2µg) per mouse.

4.4.2 Production and assessment of antisera

4.4.2.1 Antiserum assessment

4.4.2.1.1 Potency

The ED₅₀ was estimated at 564µg (480µg-640µg) and 414µg (320µg-520µg) of IgG per mouse for mice tested with *V.latastei* and a mixture (1:1:1) of *V.a.ammodytes*, *V.b.berus* and *V.a.aspis* venoms respectively.

4.4.2.1.2 Binding studies

There are 12, 9, 12 and 11 g/L of specific antibodies in the *V.latastei* antisera that bind respectively to solid phases coupled with *V.latastei*, *V.a.ammodytes*, *V.a.aspis* and

	Description	<i>V.latastei</i> venom	<i>V.ammodytes</i> , <i>V.berus</i> and <i>V.aspis</i> venom mixture (1:1:1)
Micrograms of immunoglobulin or immunoglobulin fragment per mouse (µg)	<i>V.latastei</i> antiserum	564 (480-640)	414 (320 -520)
	<i>V.latastei</i> antivenom (From Serum)	393 (350-430)	181 (140-220)
	Zagreb antivenom	836 (510-1220)	460 (410 – 510)
	<i>V.latastei</i> antivenom (From Blood)	576 (470 -670)	Not carried out

Table 4.1 Summary ED₅₀ results .All results are quoted with 95% confidence limits in parenthesis and the various amounts of immunoglobulin/immunoglobulin fragment were premixed with two times the LD₅₀ of the relevant venom

V.b.berus venom (Figure 4.2).

4.4.3 Production and assessment of antivenom from antisera

4.4.3.1 Antivenom assessment

4.4.3.1.1 Purity

SDS-PAGE assessment of serum digested and purified using the method adapted from Jones and Landon (2002), resulted in a dominant band of satisfactory purity corresponding to the molecular weight of $F(ab')_2$ (Figure 4.3d). The effects of diafiltration and anion exchange in purifying the serum digest (Figure 4.3) are not apparent on SDS-PAGE gels because impurities run off the gels under the conditions used.

4.4.3.1.2 Potency

The ED_{50} was estimated at $393\mu\text{g}$ ($350\mu\text{g}$ - $430\mu\text{g}$) and $181\mu\text{g}$ ($140\mu\text{g}$ - $220\mu\text{g}$) of $F(ab')_2$ per mouse, for mice tested with antivenom against *V.latastei* and a mixture (1:1:1) of *V.ammodytes*, *V.berus* and *V.aspis* venoms, respectively.

4.4.4 Assessment of Zagreb antivenom

4.4.4.1 Purity

Two dominant bands can be observed at approximately 97kDa and approximately 84kDa. There is also a band at the top of the gel indicating the possible presence of some undigested whole immunoglobulin (Figure 4.3).

4.4.4.2 Potency

Estimated at $836\mu\text{g}$ ($510\mu\text{g}$ - $1220\mu\text{g}$) and $460\mu\text{g}$ ($410\mu\text{g}$ - $510\mu\text{g}$) of immunoglobulin fragment per mouse, for mice tested with *V.latastei* and a mixture (1:1:1) of *V.ammodytes*, *V.berus* and *V. aspis* venoms respectively.

4.4.5 Investigation of conditions required for making antivenom from blood

4.4.5.1 pH

After one hour of digestion at pH 2.5 (Figure 4.4a), there remains only a faint band corresponding to IgG (approx 160,000 MW) and this has virtually disappeared after four

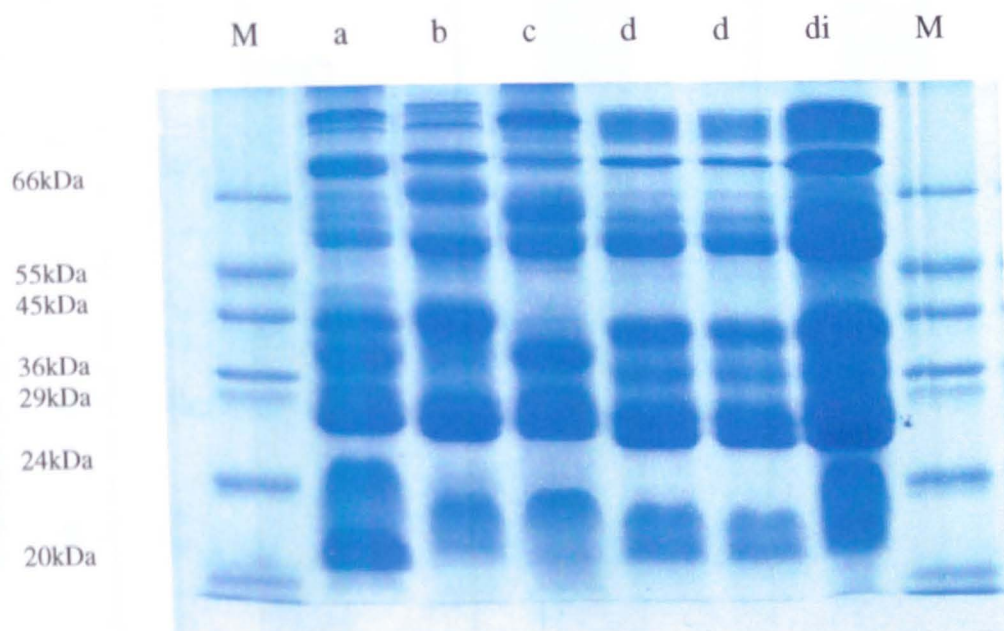


Figure 4.1 Biophysical comparison by 12% non-reducing SDS-PAGE of the venoms of (a) *V.b.berus* (b) *V.a.ammodytes* (c) *V.a.aspis* and (d) *V.latastei*. (di) is an increased (x4) sample load of (d). M are molecular weight markers.

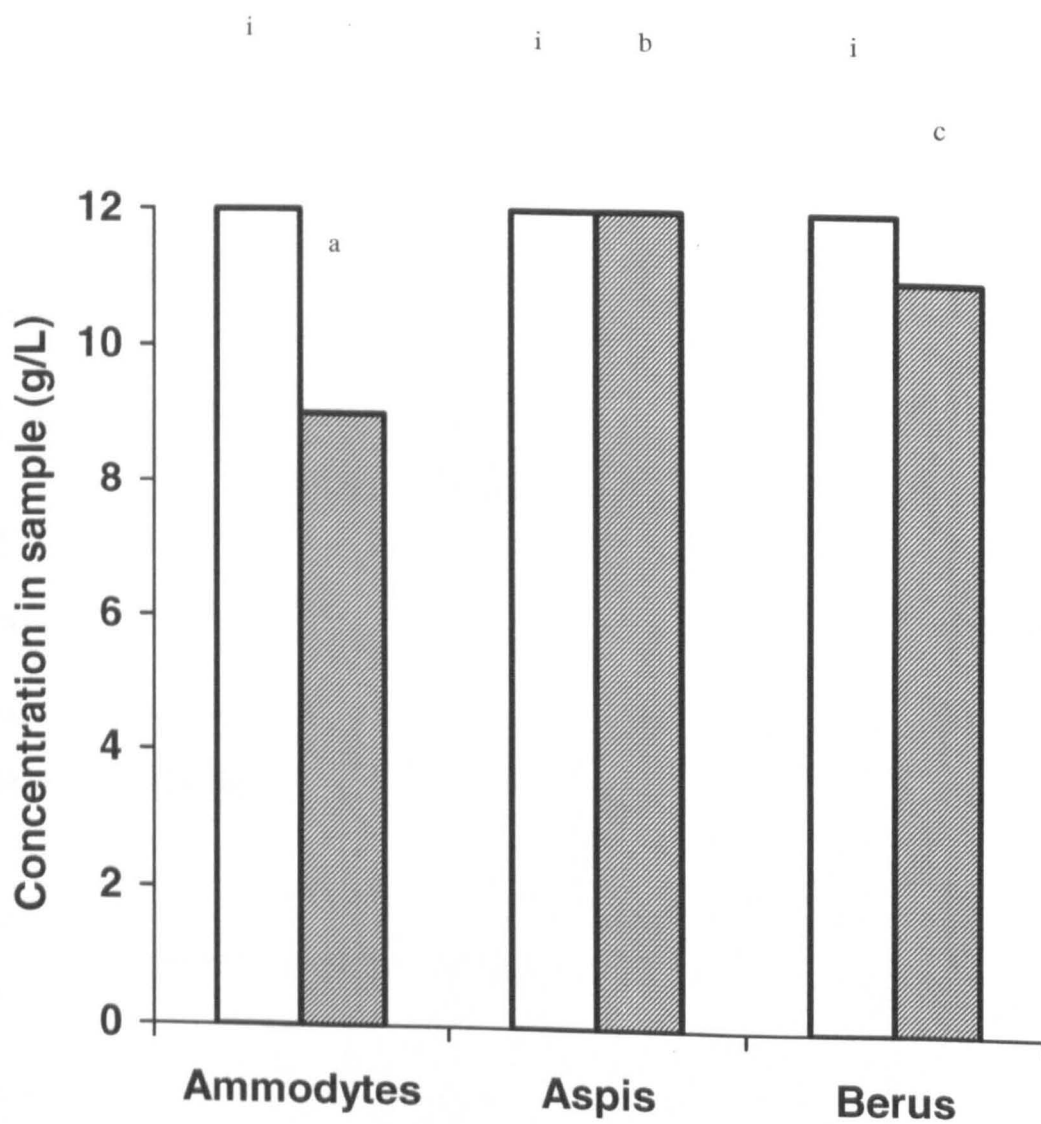


Figure 4.2 Estimation of specific antibody concentrations in *V. latastei* antisera that bind to *V. latastei* (i), *V. a. ammodytes* (a), *V. a. aspisp* (b) and *V. b. berus* (c) venom solid phases respectively

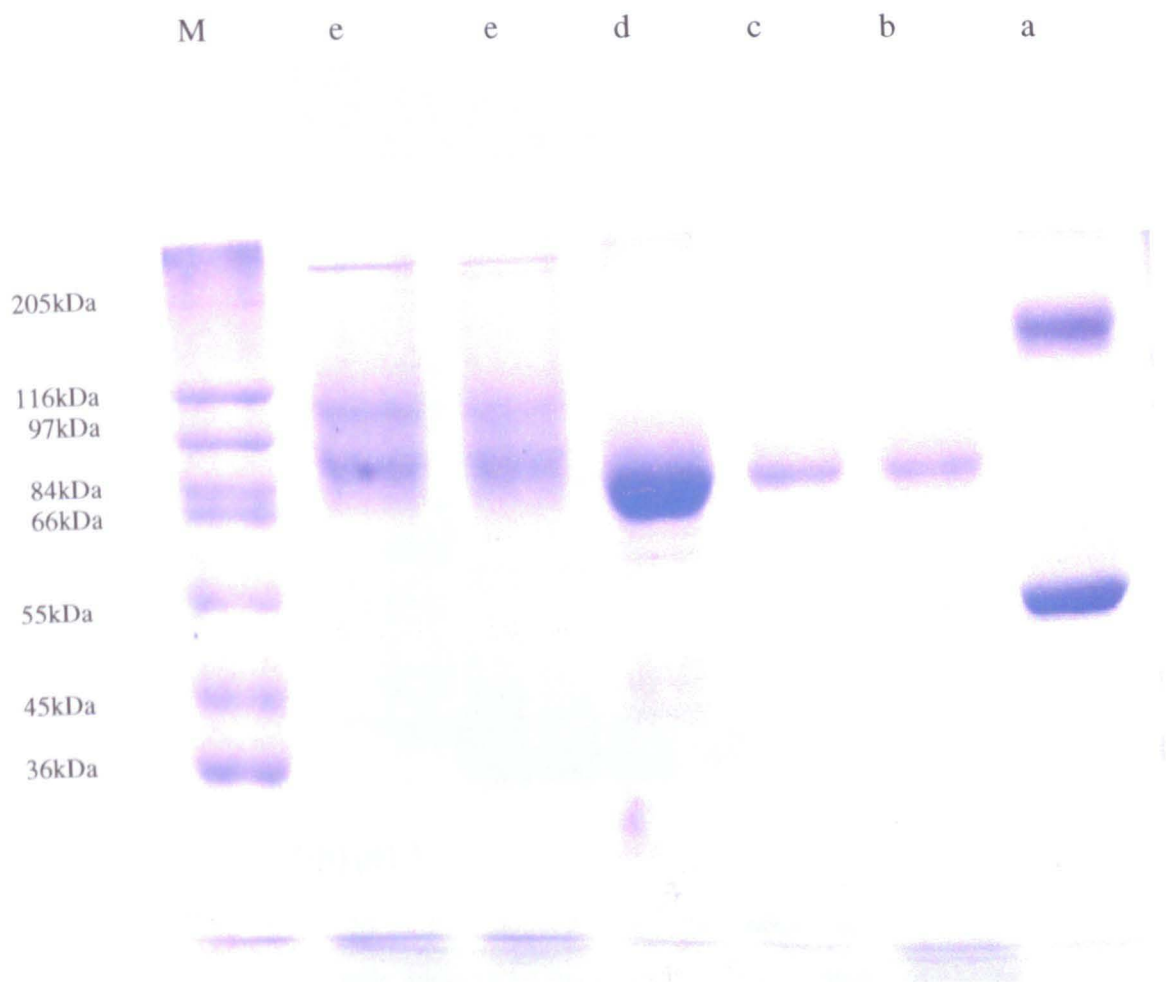
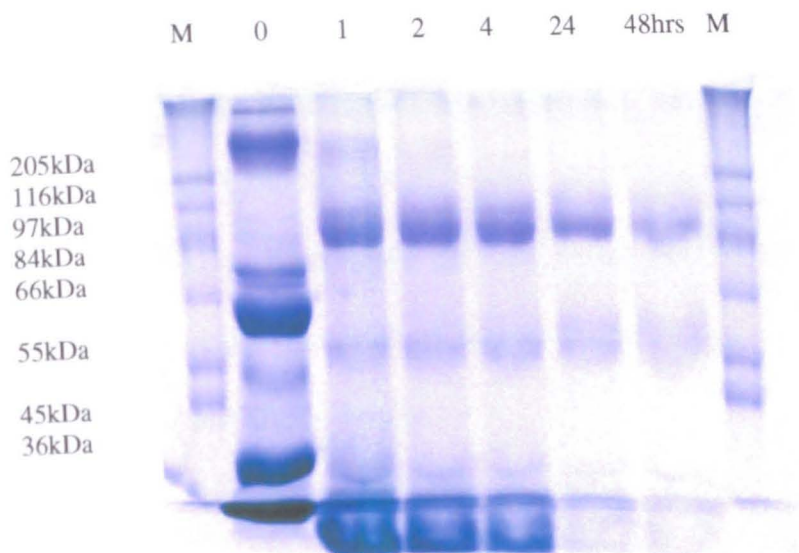
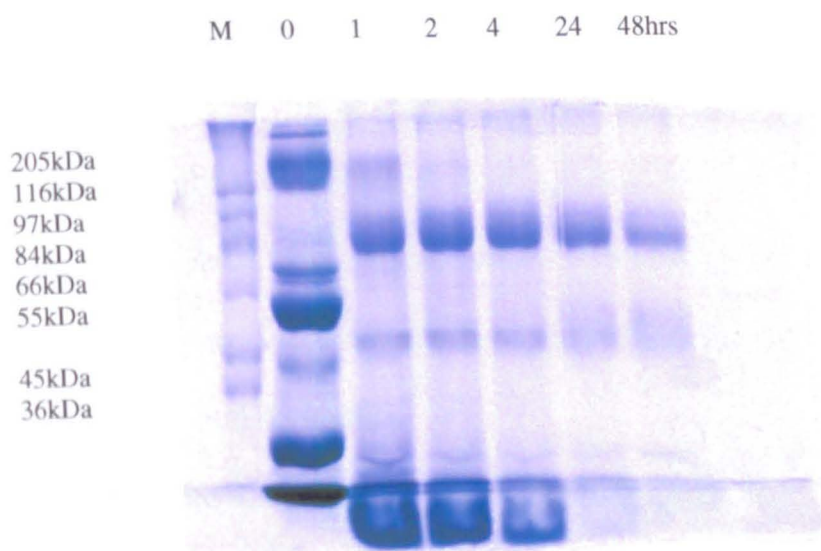


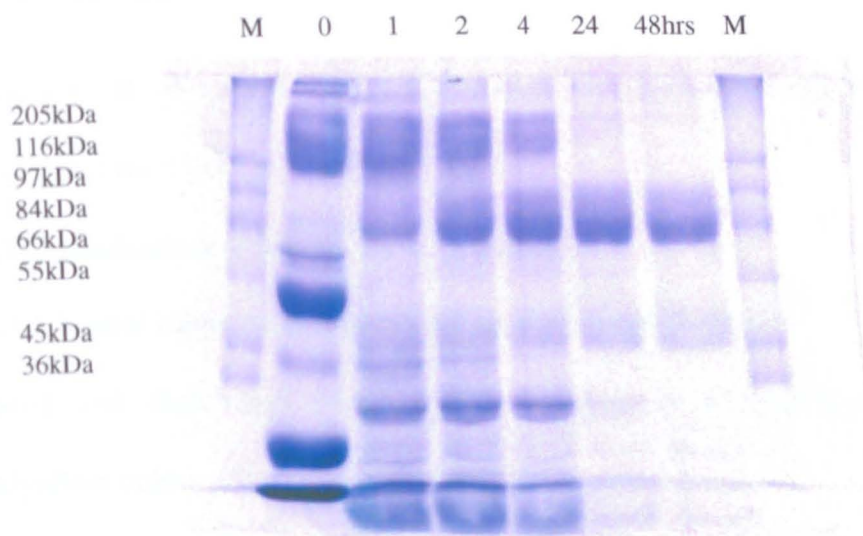
Figure 4.3 Monitoring by SDS-PAGE of serum before (a) and after its direct digestion with pepsin (b) and further processing with centrifugation and diafiltration (c) followed by ion exchange chromatography (d). Zagreb antivenom (e). M are molecular weight markers.



(a) pH 2.5



(b) pH 3



(c) pH 3.5

Figure 4.4 The effect of pH on the pepsin digestion of ovine blood

hours. Concomitantly, a dominant band appears corresponding to the molecular weight of $F(ab')_2$ (Jones and Landon, 2002), with a dispersed area of staining corresponding to approximately 45kDa. Both bands get fainter with time.

Low molecular weight material of less than 36kDa present in the original sample has virtually disappeared after 4 hours.

At pH 3 (Figure 4.4b) the results are similar to that described above, except that after 48 hours the band corresponding to $F(ab')_2$ and the 45kDa band are less faint than that obtained for the same time interval at pH 2.5.

At pH 3.5 (Figure 4.4c) the band corresponding to IgG does not disappear until 24 hours with a dominant band corresponding to $F(ab')_2$ appearing over the same time period. Also, other breakdown products of molecular weight intermediate in size between 55 and 36kDa can be observed alongside the already described dispersed area of approximately 45kDa. The latter is present until 48 hours.

Digestion at pH 4 and 4.5 produced results of no practical value to this work and have not been included.

4.4.5.2 Pepsin mass

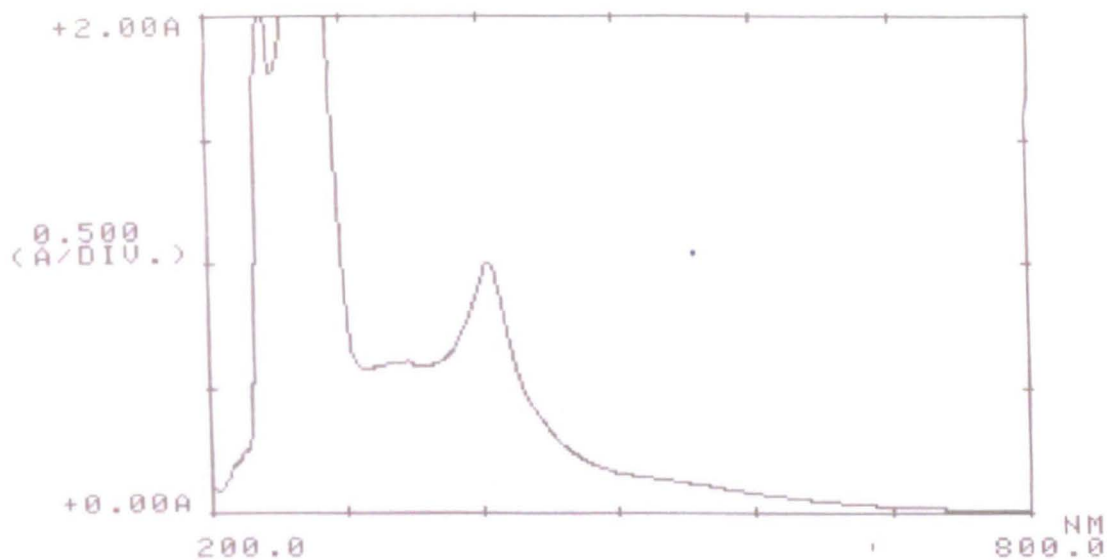
Digestion at pH 3.5 with 0.5 and 1% (w/w) pepsin produced results of no practical value to this work and thus have not been included.

4.4.5.3 Optimization of ion-exchange conditions

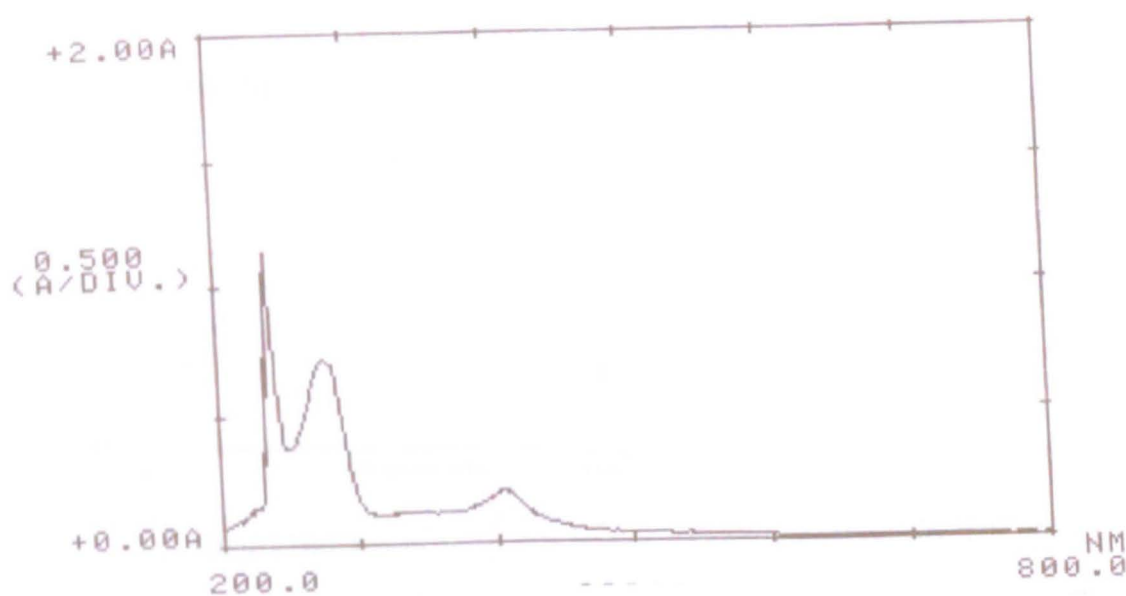
4.4.5.3.1 Visual inspection and scanning spectrophotometry

Digested and diafiltered ovine blood resulted in a $F(ab')_2$ product that exhibited green/yellow colouration by visual inspection.

Scanning spectrophotometry confirmed that there was absorbance (Figure 4.5a and b) around 400nm as was expected from the visual inspection. Chromatographic separations by anion exchange with eluant buffer containing decreasing concentrations of NaCl (Figures 4.6 a , b, c and d), that is 150, 80, 40 and 20mM, showed a maximum peak at 400nm

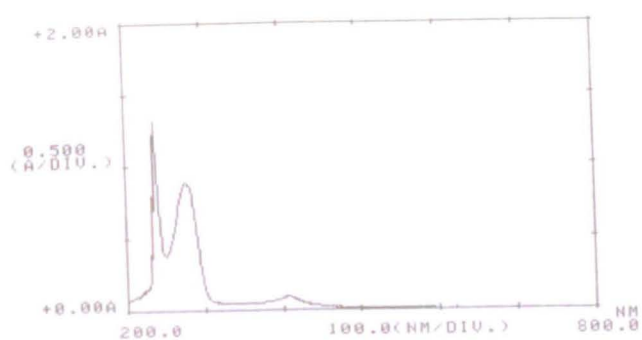


(a) Digested and diafiltered (histidine buffer, containing 150mM NaCl) ovine blood

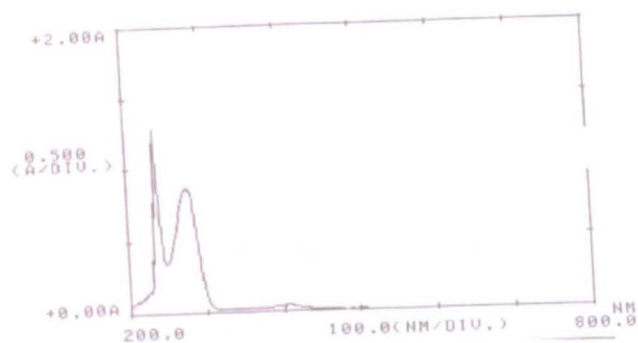


(b) Digested and diafiltered ovine blood diluted 1:5

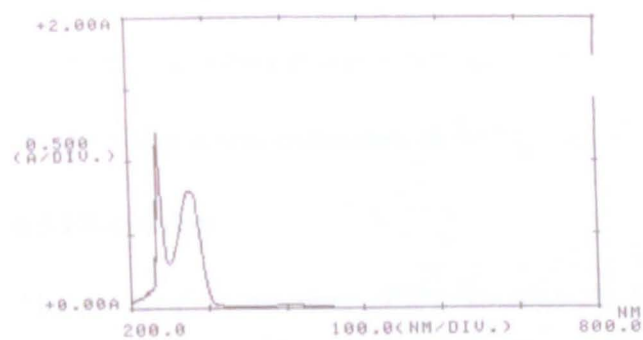
Figure 4.5 Scanning (200nm–800nm) spectrograph of pepsin digested and diafiltered ovine blood



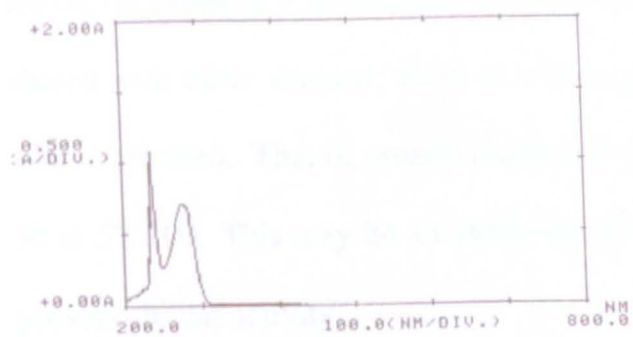
(a) 150mM NaCl



(b) 80mM NaCl



(c) 40mM NaCl



(d) 20mM NaCl

Figure 4.6 Scanning (200nm-800nm) spectrograph of pepsin digested, diafiltered and anion exchanged ovine blood in histidine buffer containing 20,40,80 and 150mM NaCl respectively

4.4.5.3.2 Purity

Digested and diafiltered (histidine buffer, containing 20mM NaCl) ovine blood resulted in a dominant band (Figure 4.7a) corresponding to the molecular weight of F(ab')₂ with two other fainter bands, one of molecular weight approximately of 116kDa and the other of approximately 45kDa. After anion exchange purification with buffers containing 150, 80, 40 and 20mM NaCl the 116kDa band virtually disappeared from the eluted F(ab')₂ peak with all NaCl concentrations whereas the 45kDa band was retained (Figure 4.7 b, c, d and e).

4.4.5.4 Potency of antivenom produced from blood

Estimated at 576µg (470µg-670µg) of F(ab')₂ per mouse for mice tested with *V.latastei* venom.

4.4.5.5 Assessment of recovery of F(ab')₂ prepared from blood and serum

For 30mL of serum it was estimated at 480mg. For 43mL of blood processed with 20 and 40mM NaCl it was estimated at 307mg and 299mg respectively.

4.5 Discussion

The physical assessment of the venoms carried out by SDS-PAGE suggests that although there are some proteins bands observed on SDS-PAGE that are specific to each venom tested, for example *V.ammodytes* venom has an approximate 66kDa protein band that is not shared with other venoms; there is a large number of bands that can be found in all the venoms studied. That is, protein bands corresponding to approximate molecular weights of 30 to 57 kDa. This may be an indication that the components of the venoms studied may possess similar activity.

A venom mixture of *V.b.berus*, *V.a.aspis* and *V.a.ammodytes* in 1:1:1 proportion is approximately two times more lethal than *V.latastei* venom as determined by LD₅₀. The binding studies indicate that there is a high concentration of specific antibodies in *V.latastei* antisera that bind to components in *V.a.ammodytes*, *V.a.aspis* and *V.b.berus* venoms as well

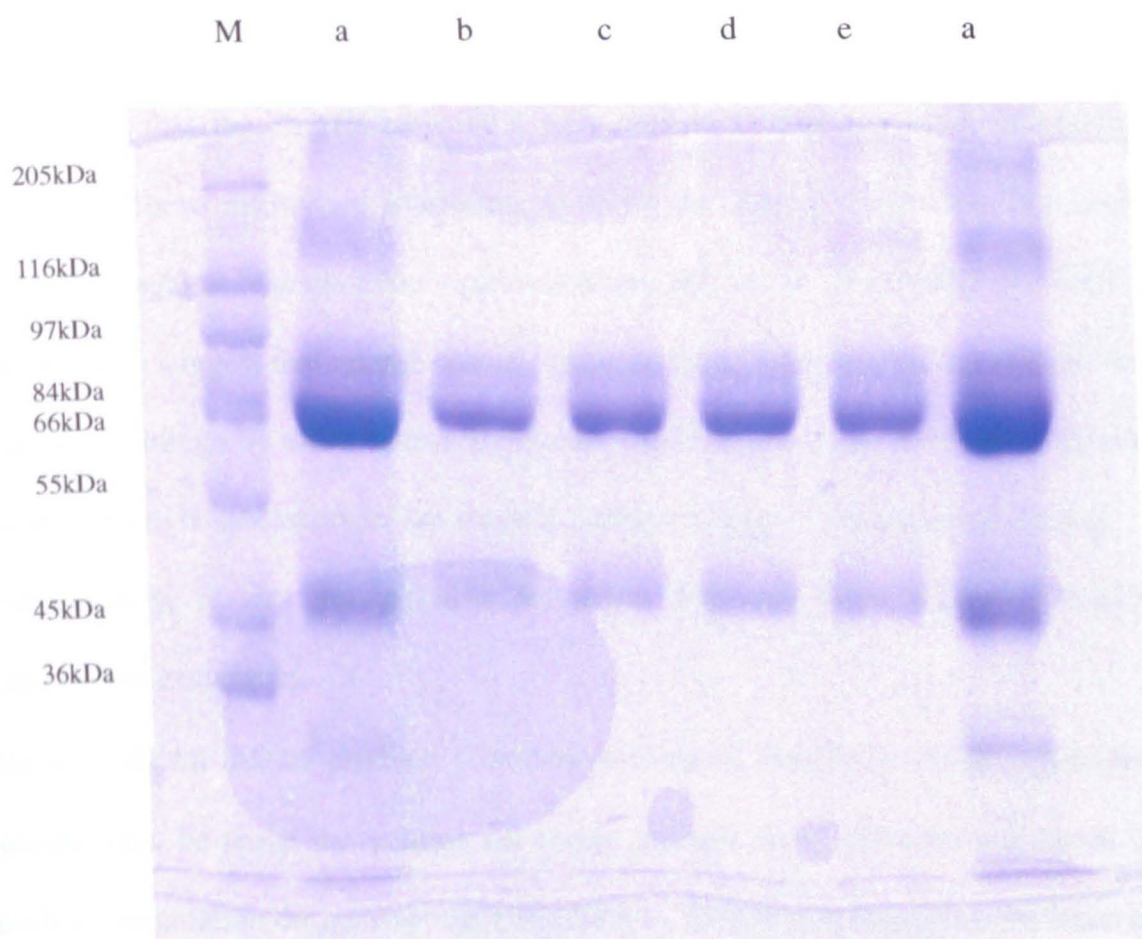


Figure 4.7 SDS-PAGE comparison of digested and diafiltered (histidine buffer, containing 20mM NaCl) ovine blood without anion exchange (a) and with anion exchange (histidine buffer, containing 150mM (b), 80mM (c), 40mM (d) and 20mM (e) NaCl)

as *V.latastei* venom. This result together with the observation that there is a large overlap of protein bands from venoms assessed by SDS-PAGE lends support to the suggestion that it is not necessary to use specific antisera to treat *V.l.latastei* envenomings.

We have shown that it is practicable on the bench scale to produce a specific ovine F(ab')₂ antivenom of acceptable purity and potency for *V.latastei* from serum employing the enhanced pepsin digestion method described by Jones and Landon, (2002). Also, we have shown that this antivenom is approximately twice as potent as the Zagreb antivenom in an ED₅₀ mouse model when tested against *V.latastei* venom and a mixture of European *Vipera* venoms and that the Zagreb antivenom may contain undigested whole immunoglobulin molecules. These results are interesting because the Zagreb antivenom has been raised specifically against *V.ammodytes* venom and our antivenom specifically against *V.latastei* venom. They suggest that an antivenom produced from *V.latastei* antisera could be used to treat envenomings by the common European *Vipera* species and lends support to the fact that it may not be necessary to use specific antisera to treat *V.latastei* envenoming.

Unfortunately, it was not possible to obtain Viperfav from Pasteur-Mérieux for a comparative evaluation.

We have shown that to produce *V.latastei* antivenom directly from blood the processing volume must be twice the volume for serum because more concentrated blood solutions result in coagulation during overnight incubation. The pepsin mass must be twice that used for serum over the same digestion time interval to cope with the extra protein content contributed by the haemoglobin, viz an extra 100g/L. The use of a comparable pepsin mass to that used for serum resulted in incomplete digestion of IgG. The pH must be 3.5 as pH 2.5, and 3 respectively lead to degradation of the main F(ab')₂ band within the time interval under investigation and a pH of 4 and 4.5 result in incomplete digestion of IgG and other serum proteins within the same time interval. We have shown that the anion exchange protocol in Jones and Landon (2002), which included the use of a histidine buffer

containing 150mM NaCl, is not optimal for processing $F(ab')_2$ from blood in that it results in antivenom containing a yellow coloured impurity. We have been able to produce a colourless product by reducing the NaCl concentration to 20mM, but this resulted in only approximately fifty percent of the recovery achieved using serum. To improve on this recovery in the future it may be necessary to explore other ways of removing the yellow coloured impurity. For example, filtration strategies or positive ion-exchange chromatography where the product binds the ion-exchange column and the impurity travels through it depending on the choice of conditions.

Essentially the reaction conditions for the direct digestion of blood are the same as for direct digestion of serum. The only differences are that a dilution factor of 1:2 is implemented for serum whereas a minimum dilution factor of 1:3 is needed for blood and further processing with ion-exchange chromatography necessitates a large reduction of the NaCl content of the buffer.

We have also shown that there is a residual impurity seen on SDS-PAGE gels from the digestion of blood that may have originated from over-digestion of $F(ab')_2$ to Fab fragments due to the extra pepsin requirement or to a by-product of blood digestion that is resistant to pepsin. None-the-less the potency of the resultant $F(ab')_2$ from blood is comparable to that produced from serum.

CHAPTER 5: FINAL DISCUSSION AND CONCLUSIONS

The customary method of refining and concentrating antitoxins during the early part of the last century entailed fractional precipitation of the different serum proteins by the addition of inorganic salt (Gibson, 1905). Later Parfentjev (1936) discovered that, by the use of proteolytic enzyme digestion, it was possible to purify and concentrate the antitoxin further than was possible by salt fractionation alone. This was the last significant advance in the manufacture of antitoxins until the late 1960's when a group in Boston raised antibodies directed against the cardiac glycosides and showed that they could reverse the clinical manifestations of digoxin overdose (Butler and Chen, 1967). Three important innovations were incorporated into the development of a subsequent therapeutic product (Digibind) manufactured by Wellcome. First, sheep were preferred to horses; second, the immunoglobulins were cleaved with papain to produce Fab; and third, only Fab directed specifically against cardiac glycosides were used after separation from the much larger amounts of non-specific Fab by affinity chromatography. Such products have been given to many hundred patients and have an enviable reputation as regards both efficacy and safety (Antman et al. 1990).

We have summarised the steps involved in making an antivenom (Figure 1.5, p.23). This work has been concerned primarily in the areas of immunising and bleeding, laboratory assessment, initial extraction and purification. The other areas have not been considered and will not be discussed further.

Appropriate assessment methods are essential in comparative studies of the humoral response in different animal species and in following extraction and purification procedures.

Kowalczyk et al.(1985) reported that it was possible to recover approximately 120mg of IgY per egg; whilst Losch et al.(1986) reported values ranging from 40 to 500mg IgY and

McLaren et al.(1994) approximately 130mg per egg. Our studies, based on a fluoroimmunoassay developed for these purposes, gave recoveries of approximately 145mg of IgY per fresh egg using the extraction method of choice. The fluoroimmunoassay may also have application in documenting the immunological status in the chicken by monitoring total IgY concentrations in serum in response to immunisation.

The manual ELISA method, although very widely used for assessment of antisera and antivenoms (Theakston et al.1977; Sjostrom et al. 1996) has many difficulties and disadvantages. These include the need for specialised and expensive equipment (plate washers, plate readers, multichannel pipettes); non reusable materials and consumables(microtitre plates, venom for coating, second antibody for detection); and the use of an enzymatic detection step, often with potentially carcinogenic substrates requiring special disposal. The method is multistep and labour intensive which, together with the use of a temperature dependent enzyme detection step, results in marked imprecision of titre values. Typically 20 – 50% coefficients of variation have been observed in various in-house manual ELISA systems used by myself and co-workers. A further drawback of manual ELISA is that it provides results in arbitrary titre units, which cannot be related directly to the actual antibody content of the antisera.

A small scale affinity purification method was optimised and characterised during this work whose only requirements were small venom affinity columns and a UV spectrophotometer. Compared with ELISA, the advantages of SSAP are the use of simpler, less expensive instrumentation (important for the third world); simpler reagents, with the capability to reuse venom coupled columns (unlike coated ELISA plates which must be discarded after a single use); a simple procedure with fewer steps, leading to improved precision (CV about 6%); and determination of actual physical quantities of specific antibodies in g/L.

Chicken egg yolk is a potential source of inexpensive antivenoms, provided simple, inexpensive methods of extracting and purifying its IgY can be developed.

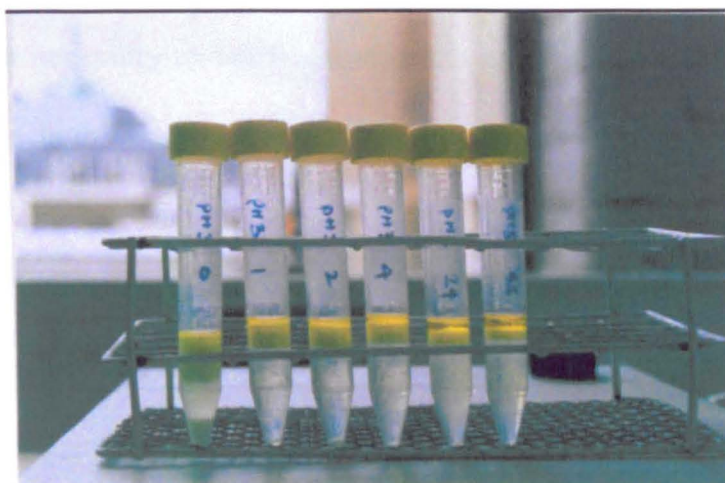
The purification of IgY from egg yolk was divided into two distinct areas, namely lipoprotein removal (step1) and IgY separation (step 2). It was shown that caprylic acid was the best lipoprotein removal method from a panel of candidate methods frequently used in practice. This finding was unexpected because caprylic acid has been described in the literature only once for this application (McLaren et al. 1994) and has never been promoted. This may be because it produces an infranatant aqueous layer which is difficult to handle (Figure 5.1a). We have been able to scale up the use of caprylic acid by the use of air filled blood bags (Figure 5.1b) which can be centrifuged and punctured to obtain the infranatant layer without difficulty. This innovation enabled the processing of large numbers of eggs (to date, up to approximately 30) with satisfactory purity and recovery (results not reported here). No innovations were made in step two of the purification.

It was disappointing to find that total IgY did not protect the mice in the relevant potency assessment and that we had to affinity purify the latter to produce an effective antivenom. Other investigators (Almeida et al. 1998; Maya et al. 2002) have managed to produce effective antivenoms without affinity purification and thus further studies are indicated.

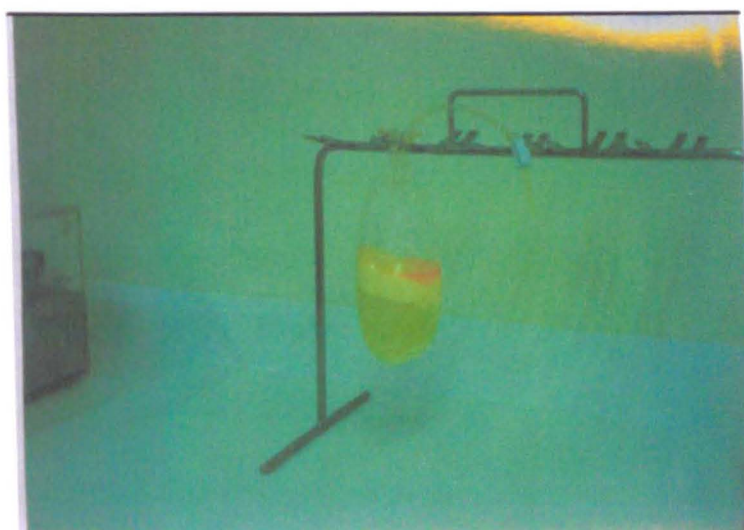
Possible areas for investigation are: dose/response studies. Thus the present studies used the venom doses typically used in sheep protocols and more studies are required to show the ideal venom dose regimen for hens, the route (i.m. pectorally) and the frequency of immunisation.

As early as 1936 Parfentjev recognized the potential of proteolytic enzyme digestion for the removal of unwanted proteins. Pope (1938) used these findings as the basis of a peptic digestion method for the purification of antitoxic plasmas and the latter has been updated by Jones and Landon (2002).

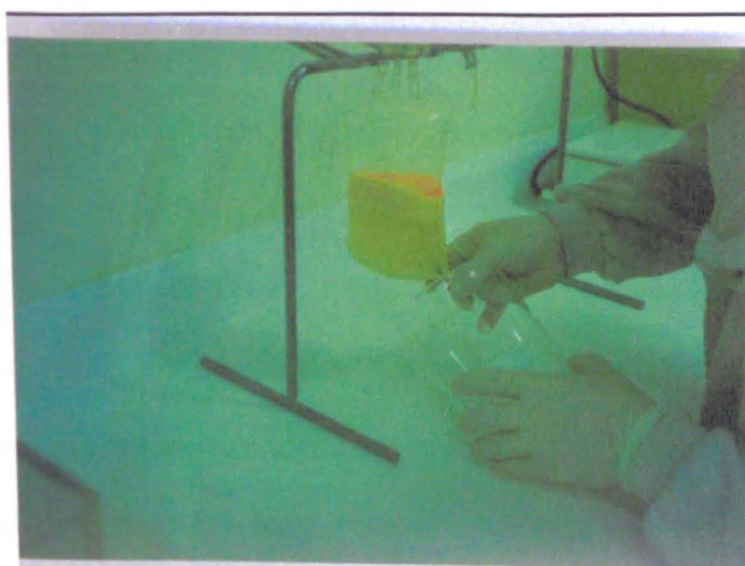
In this work we have used pepsin as described by Akita and Nakai (1993) to make IgY Fab'



(a) Laboratory scale. Infranatant aqueous layer



(b) Scale up. Inflated centrifuged blood bag



(c) Punctured bag to obtain infranatant layer

Figure 5.1 Laboratory and scale up preparation of caprylic acid extract

fragments from IgY processed from chicken eggs. We have gone a step further and have shown that it is not necessary to salt fractionate the caprylic acid extracted IgY prior to its digestion. This has both simplified the procedure and resulted in improved recovery of Fab'.

To my knowledge the direct digestion of whole blood with pepsin has only been described by Harms (1948), who noted that the product was less pure (on the basis that it was darker in colour) but with better recovery (albeit only moderately better) than product produced from plasma. He concluded that there was no advantage to be gained by processing whole blood and didn't describe his procedure in detail. Our contribution here has been to obtain a pure, colourless product from direct blood digestion with pepsin by a combination of diafiltration and anion exchange chromatography. The latter required careful selection of the salt strength of the ion exchange buffer. These techniques had not been invented in 1948 and thus were not available to Harms. Diafiltration and ion exchange chromatography were used by Jones and Landon (2002) to remove small molecular weight products of digestion and aggregates, respectively after pepsin digestion of whole serum. We have extended its use here to whole blood digestion to remove coloured products.

At present our findings agree with those of Harms (1948). That is that no major advantage is gained by processing whole blood over serum other than missing out the blood clotting step. None the less we cannot rule out some use for blood digestion in the future.

Overall, the use of pepsin to eliminate unwanted proteins has made it possible to reduce the total number of processing steps necessary to produce immunoglobulin fragments. Thus, in egg yolk and serum processing we have been able to do away with the salt fractionation step and in blood processing we have been able to remove both the clotting and salt fractionation steps.

BIBLIOGRAPHY

- Akita EM, Nakai S. Immunoglobulins from egg yolk: Isolation and purification. *J Food Sci* 1992;57:629-34.
- Akita EM, Nakai S. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic E.coli strain. *J Immunol Methods* 1993;160:207-14.
- Akita EM, Nakai S. Production and purification of Fab' fragments from chicken egg yolk immunoglobulin Y. *J Immunol Methods* 1993;162:155-64.
- Almeida CM, Kanashiro MM, Rangel Filho FB, Mata MF, Kipni TL, DaSilva WD. Development of snake antivenom antibodies in chickens and their purification from yolk. *Vet Rec* 1998;143:579-84.
- Angulo Y, Estrada R, Gutierrez JM. Clinical and laboratory alterations in horses during immunization with snake venoms for the production of polyvalent (Crotalinae) antivenom. *Toxicon* 1997;35:81-90.
- Antman EM, Wenger TL, Butler VP, Jr., Haber E, Smith TW. Treatment of 150 cases of life-threatening digitalis intoxication with digoxin-specific Fab antibody fragments. Final report of a multicenter study. *Circulation* 1990;81:1744-52.
- Ariaratnam CA, Meyer WP, Perera G et al. A new monospecific ovine Fab fragment antivenom for treatment of envenoming by the Sri Lankan Russell's viper. A preliminary dose-finding and pharmacokinetic study. *Am J Trop Med Hyg* 1999;61:259-65.
- Arnold EN, Burton JA. *A field guide to the reptiles and amphibians of Britain and Europe*. London: Collins,1992.
- Bellairs R. The structure of the yolk of the hen's egg as studied by electron microscopy. *J Biophys Biochem Cytol* 1961;11:207-25.
- Bentley GA. A structural view of immune recognition by antibodies. *Res Immunol* 1994;145:31-45.
- Bernardi G, Cook WH. An electrophoretic and ultracentrifugal study on the proteins of the high density fraction of egg yolk. *Biochem Biophys Acta* 1960;44:86-96.
- Bomford R. Adjuvants for anti-parasite vaccines. *Parasitol Today* 1989;5:41-46.

Boulenger GA. Poison apparatus – Different kinds of venom. In *The Snakes of Europe*. Methusen & Co. Ltd of London, 1913,35-39.

Branden C, Tooze J. Recognition of foreign molecules by the immune system. In *Introduction to Protein Structure*. New York and London: Garland Publishing,1991, 179-99.

Brandly CA, Moses HE, Jungherr EL. Transmission of antiviral activity via the egg and the role of congenital passive immunity to Newcastle disease in chickens. *Poult Sci* 1946;25:397-98.

Burley RW, Cook WH. Isolation and composition of avian egg yolk granules and their constituent α and β lipovitellins. *Can J Biochem Physiol* 1961;39:1295-307.

Burley RW. Lipoproteins. In Johnson AR, Davenport JB, eds. *Biochemistry and Methodology of Lipids*. NewYork: Wiley Interscience, 1971, 85-106.

Burrin DH. Spectroscopic techniques. In Wilson K, Goulding KH, eds. *A Biologist's Guide to Principles and Techniques of Practical Biochemistry*. Suffolk: Edward Arnold, 1986, 270-312.

Burrin DH. Immunochemical techniques. In Wilson K, Goulding HK, eds. *A Biologist's Guide to Principles and Techniques of Practical Biochemistry*. Suffolk: Edward Arnold,1986, 116-52.

Butler VP, Jr., Chen JP. Digoxin-specific antibodies. *Proc Natl Acad Sci* 1967;57:71-78.

Carroll SB, Thalley BS, Theakston RDG, Laing G. Comparison of the purity and efficacy of affinity purified avian antivenoms with commercial equine crotalid antivenoms. *Toxicon* 1992;30:1017-25.

Calmette A. Contribution à l'étude du venin des serpents. Immunisation des animaux et traitement de l'envenimation. *Ann Inst Pasteur* 1894;VIII:275-291.

Chard T. *An introduction to radioimmunoassay and related techniques*, 4 ed. Elsevier,1990.

Chatterjee SC, Dass B, Devi P. A comparative study on different methods of hyperimmunization of horses for the preparation of polyvalent anti-snake venom serum. *Indian J Med Res* 1968;56:678-85.

Cheng AC, Winkel K. Call for global snake-bite control and procurement funding. [letter; comment.]. *Lancet* 2001;357:1132.

Chippaux JP, Williams V, White J. Snake venom variability: Methods of study, results and interpretation. *Toxicon* 1991;29:1279-303.

Chippaux JP, Goyffon M. Venoms, antivenoms and immunotherapy. *Toxicon* 1998;36:823-46.

Chippaux JP, Goyffon M. Les morsures accidentelles des serpents en France metropolitaine. *Presse Med* 1989;18:794.

Chotwiwatthanakun C, Pratanaphon R, Akesowan S, Sriprapat S, Ratanabanangkoon K. Production of potent polyvalent antivenom against three elapid venoms using a low dose, low volume, multi-site immunization protocol. *Toxicon* 2001;39:1487-94.

Christensen PA. The preparation and purification of antivenoms. *Mem Inst Butantan Simp Internac* 1966;33:245-50.

Claud B, Chague A, Tourret J. Morsures de vipères. *Cah Anesthesiol* 1989;37:259-64.

Cook WH. Egg Quality. In Carter TC, ed. *A Study of the Hen's Egg*. Oliver and Boyd: Edinburgh, 1968,109-32.

Coulter AR, Harris RD, Sutherland SK. Clinical laboratory: Enzyme immunoassay for the rapid clinical identification of snake venom. *Med J Aust* 1980;1:433-35.

Curd J, Smith TW, Jatton J-C, Haber E. The isolation of digoxin-specific antibody and its use in reversing the effects of digoxin. *Proc Nat Acad Sci* 1971;68:2401-6.

Debra JJ, Donald Capra J. Immunoglobulins: Structure and Function. In Paul WE, ed. *Fundamental Immunology*. New York: Raven Press, 1984, 131-165.

Detrait J, Naulleau G, Saint-Girons H. Contribution à l'étude du venin de *V. latastei* Bosca,1878. *Munibe* 1983;35:81-86.

Dos Santos MC, D'Imperio Lima MR, Furtado GC, Colletto GM, Kipnis TL, Dias DS. Purification of F(ab')₂ anti-snake venom by caprylic acid: A fast method for obtaining IgG fragments with high neutralization activity, purity and yield. *Toxicon* 1989;27:297-303.

Edwards R. Historical development of immunoassays. In *Immunoassay an introduction*. London: William Heinemann Medical Books, 1985, 1-11.

Ekins RP. Estimation of T4 in human plasma by an electrophoretic technique. *Clin Chim Acta* 1960; 5:453 -9

European Commission report. Poison centres:Collection of the annual reports 1994 Analysis and synthesis. SOC 95 201617 05FO3. 1996. Bruxelles, Europlus s.a.

Fischer M, Hlinak A. An ELISA for the quantification of chicken immunoglobulin(IgY)in various liquid media. *ALTEX* 1996;13:179-83.

Fraser R, Jukes TH, Branion HD, Halpern KD. The inheritance of diphtheria immunity in ducks. *J Immunol* 1933;26:437-46.

Gibson RB. The concentration of antitoxin for therapeutic use. *J Biol Chem* 1905;1:161-70.

Gilbert AB. *Advances in Reproductive Physiology*. London: Logos Press, 1967.

González D. Snakebite problems in Europe. In Tu AT, ed. *Handbook of Natural Toxins, Reptile Venoms and Toxins*. New York: Marcel Dekker, 1991, 687- 751.

Grandgeorge M, Veron JL, Lutsch C et al. Preparation of improved F(ab')₂ antivenoms. An example: New polyvalent European viper antivenom (equine). In Bon C, Goyffon M, eds. *Envenomings and their treatments*. Paris: Foundation Marcel Merieux, 1996,161-72.

Harms AJ. The purification of antitoxic plasmas by enzyme treatment and heat denaturation. *Biochem J* 1948;42:390-397.

Harris JB. Toxic constituents of animal venoms and poisons. *Adv Drug React Ac Pois Rev* 1982;1:65.

Ho M, Warrell MJ, Warrell DA, Bidwell D, Voller A. A critical reappraisal of the use of enzyme-linked immunosorbent assays in the study of snake bite. *Toxicon* 1986;24:211-21.

Huber R. Three-dimensional structure of antibodies. *Behring Inst Mitt* 1984;76:1-14.

Jenner E. An enquiry into the causes and effects of the *Variolae vaccinae*. Sampson Low, No.7 Deawick Street, Soho, London.1798; 1-85.

Jensenius J, Andersen I, Hau J, Crone M, Koch C. Eggs:Conveniently packaged antibodies.Methods for purification of yolk IgG. *J Immunol Methods* 1981;46:63-68.

Jones RG, Landon J. Enhanced pepsin digestion: A novel process for purifying antibody F(ab')₂ fragments in high yield from serum. *J Immunol Methods* 2002;263:57-74.

- Jungling A, Wiedemann V, Kuhlmann R, Erhard M, Schmidt P, Losch U. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. IV. In vitro studies on protective effects against adhesion of enterotoxogenic *Escherichia coli* to isolated enterocytes. *Zentralbl Veterinarmed [B]* 1991;38:373-81.
- Kirkpatrick CH. Allergic histories and reactions of patients treated with digoxin immune Fab (ovine) antibody. The Digibind Study Advisory Panel. *Am J Emerg Med* 1991;9:7-10.
- Klaassen CD, Eaton DL. Principles of Toxicology. In Amdur MO, Doull J, Klaassen CD, eds. *Casarett and Doull's Toxicology: The basic science of poisons*. New York: Pergamon press, 1985, 12-49.
- Klemmer K. *Venomous Animals and their Venoms*. New York: Academic Press, 1968.
- Klempner F. Über natürliche Immunität und ihre Verwerthung für die Immunisierungstherapie. *Arch Expl Pathol Pharmacol* 1893;31:356-82.
- Kowalczyk K, Daiss J, Halpern J, Roth TF. Quantitation of maternal-fetal IgG transport in the chicken. *Immunology* 1985;54:755-62.
- Kuhlmann R, Wiedemann V, Schmidt P, Wanke R, Linckh E, Losch U. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. I. Immunization and antibody determination. *Zentralbl Veterinarmed [B]* 1988;35:610-616.
- Landon J, Chard T. *Therapeutic Antibodies*. London: Springer-Verlag, 1995.
- Lea CH, Hawke JC. The influence of water on the stability of lipovitellin and the effects of freezing and of drying. *Biochem J* 1952;52:105-114.
- Lesk AM, Tramontano A. Antibody structure and structural predictions useful in guiding antibody engineering. In Borrebaeck CAK, ed. *Antibody engineering: A practical guide*. New York: W.H. Freeman and Company, 1992, 1-35.
- Leslie GA, Clem LW. Phylogeny of immunoglobulin structure and function. *J Exp Med* 1969;130:1337-52.
- Liener I. The sulfhydryl proteases: Food related enzymes. In Whitaker J, ed. *Advances in chemistry series 136*. Washington DC: American Chemical Society, 1974, 202-220.
- Losch U, Schraner I, Wanke R, Jurgens L. The chicken egg, an antibody source. *Zentralbl Veterinarmed B* 1986;33:609-19.

Losonczy S, Szabó C, Kiss Z, Bárdos L. Application of an anti-HQIgY antibody for the measurement of IgY concentrations of hen's and quail's serum and yolk. *Acta Physiologica Hungarica* 1999;86:253-58.

Marquard H. Recherches statistique sur les accidents par morsure de serpents au Danemark et en Suède de 1900 à 1947. *Presse Med* 1951;59:1110.

Martin WG, Vandegaer JE, Cook WH. Fractionation of livetin and the molecular weights of the α and β components. *Can J Biochem Physiol* 1957;35:241-50.

Martin WG, Cook WH. Preparation and molecular weight of γ -livetin from egg yolk. *Can J Biochem Physiol* 1958;36:153-60.

Mattison C. *The Encyclopedia of Snakes*. London: Blandford,1995.

Maya DC, Vasantha BM, Krishnan LK. Development of viper-venom antibodies in chicken egg yolk and assay of their antigen binding capacity. *Toxicon* 2002;40:857-61.

McFerran TA. *Minidictionary for Nurses*, Third ed. Oxford: Oxford University Press,1995.

McGowen MW, Artiss JD, Strandbergh DR, Zak B. A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clinical Chemistry* 1983;29:538-542.

McLaren RD, Prosser CG, Grieve RCJ, Borissenko M. The use of caprylic acid for the extraction of the immunoglobulin fraction from egg yolk of chickens immunised with ovine α -lactalbumin. *J Immunol Methods* 1994;177:175-84.

Meyer WP, Habib AG, Onayade AA et al. First clinical experiences with a new ovine Fab Echis ocellatus snake bite antivenom in Nigeria: Randomized comparative trial with Institute Pasteur Serum (Ipser) Africa antivenom. *Am J Trop Med Hyg* 1997;56:291-300.

Nkinin SW, Chippaux JP, Pietin D, Doljansky Y, Tremeau O, Menez A. Genetic origin of venom variability: Impact on the preparation of antivenin serums. *Bull Soc Pathol Exot* 1997;90:277-81.

Parfentjev, A. I. Method for the purification of antitoxins and the like. Patent [2065196]. 1936. United States Patent.

Patterson R, Younger JS, Weigle WO. Antibody production and transfer to egg yolk in chickens. *J Immunol* 1962;89:272-78.

Persson H. Clinical toxicology of snake bite in Europe. In Meier J, White J, eds. *Handbook of clinical toxicology of animal venoms and poisons*. NewYork: CRC Press,1995, 413-32.

Persson H, Irestedt B. A study of 136 cases of adder bite treated in Swedish hospitals during one year. *Acta Med Scand* 1981;210:433-39.

Pérez MJF, Ferrieu PDM, Pequeño GB. Suero antiofidico frente a *Vipera latasti*. *Medicina Clínica* 1999;113:754-55.

Phelps T. The vipers. In *Poisonous snakes*. Poole, Dorset: Blandford Press, 1981,85-117.

Phisalix C, Bertrand G. Sur la propriete antitoxique du sang des animaux vaccines contre le venin de vipere. *C R Acad Sci* 1894;118:356-358.

Polson A, Barbara von Wechmar M. Isolation of viral IgY antibodies from yolks of immunized hens. *Immunological Communications* 1980;9:475-93.

Polson, A. Isolation of IgY from the yolks of eggs by a chloroform polyetheylene glycol procedure. *Immunological investigations* 1990;19: 253-258..

Pope CG. Disaggregation of proteins by enzymes. *Br J Exp Pathol* 1938;19:245-51.

Porter RR. The hydrolysis of rabbit gamma-globulin and antibodies with crystalline papain. *Biochem J* 1959;73:119-27.

Pourfarzaneh M, Nargessi RD. Composite polyacrolein-coated cellulose magnetisable particles: An "autoreactive" magnetisable solid-phase with superior buoyancy properties. *Clinica Chimica Acta* 1981;111:61-63.

Pozio E. Venomous snake bites in Italy: Epidemiological and clinical aspects. *Trop Med Parasitol* 1988;39:62-66.

Ramon G. Des anatoxines. *C R Acad Sci* 1924;178:1436-39.

Reid HA. Adder bites in Britain. *Br Med J* 1976;2:153-56.

Roitt IM. *Essential Immunology*, Sixth ed. London: Blackwell Scientific Publications, 1988.

Rose ME, Orlans E, Buttress N. Immunoglobulin classes in the hen's egg: Their segregation in yolk and white. *Eur J Immunol* 1974;4:521-23.

Roux P, Yersin A. Contribution a l'etude de la diphterie. *Ann Inst Pasteur* 1888;2:629-649

Rowsselot JC, Berthier JC, Roze JC, Floret D, Vidailhet M. Envenomation viperine grave. *Arch Fr Pediatr* 1991;48:589.

Rucavado A, Moreno E, Gutierrez JM. Effect of adjuvants on the antibody response of mice to *Bothrops asper* (Terciopelo) snake venom. *Braz J Med Biol Res* 1996;29:1337-40.

Russell FE, Timmerman WF, Meadows PE. Clinical use of antivenin prepared from goat serum. *Toxicon* 1970;8:63-65.

Saint Girons H. Biogeographie et evolution des Viperes europeenes. *Bull Soc Zool Fr* , 1980;103:155-66.

Salva M JA. Estudio farmacologico del veneno de la Vipera Latasttei Bosca. *Trab Inst Nac Cienc Med* , 1946;6:397-409.

Samloff IM. Pepsinogens, pepsins, and pepsin inhibitors. *Gastroenterology* 1971;60:586-604.

Sanchez EE, Galan JA, Perez JC, Rodriguez-Acosta A, Chase PB, Perez JC. The efficacy of two antivenoms against the venom of North American snakes. *Toxicon* 2003;41:357-65.

Schade R, Pfister C, Halatsch R, Henklein P. Polyclonal IgY antibodies from chicken egg yolk-an alternative to the production of mammalian IgG type antibodies in rabbits. *ATLA* 1991;19:403-19.

Schade R, Erhard M. Short introduction to hen's humoral immune system. In Schade R, Behn I, Erhard M, Hlinak A, Staak C. Eds. *Chicken Egg Yolk Antibodies, production and application*. Berlin Heidelberg: Springer-Verlag, 2001,1-8.

Schmidt P, Wiedemann V, Kuhlmann R, Wanke R, Linckh E, Losch U. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. II. In vitro studies on gastric and enteric digestion of egg yolk antibodies specific against pathogenic *Escherichia coli* strains. *Zentralbl Veterinarmed [B]* 1989;36:619-28.

Scholer H, Wuthrich W. Clinical and toxological problems in poisonous snake bites. *Schweiz Med Wochenschr* 1970;100:1761-66.

Sewell H. Experiments on the preventive inoculation of rattlesnake venom. *J Physiol* 1887;8:203-210.

Shephard CC, Hottle GA. Studies of the composition of the livetin fraction of the yolk of hen's eggs with the use of electrophoretic analysis. *J biol Chem* 1949;179:349-57.

Silverstein MA. The history of immunology. In Paul WE, ed. *Fundamental Immunology*. New York: Raven Press, 1984, 23-40.

Sjöström L, Al Abdulla IH, Rawat S, Smith DC, Landon J. A comparison of ovine and equine antivenoms. *Toxicon* 1994;32:427-33.

Sjöström L, Karlson-Stiber C, Persson H, Al Abdulla IH, Smith DC. Development and clinical application of immunoassays for European adder (*Vipera berus berus*) venom and antivenom. *Toxicon* 1996;34:91-98.

Smith DC, Reddi KR, Laing G, Theakston RDG, Landon J. An affinity purified ovine antivenom for the treatment of *Vipera berus* envenoming. *Toxicon* 1992;30:865-71.

Smith HM, Smith RB, Sawin Lewis H. A summary of snake classification (Reptilia, Serpentes). *Journal of herpetology* 1977;11:115-21.

Sriprapat S, Aeksowan S, Sapsutthipas S et al. The impact of a low dose, low volume, multi-site immunization on the production of therapeutic antivenoms in Thailand. *Toxicon* 2003;41:57-64.

Stahel E, Wellauer R, Freyvogel TA. Poisoning by domestic vipers (*Vipera berus* and *Vipera aspis*). A retrospective study of 113 patients. *Schweiz Med Wochenschr* 1985;115:890-896.

Steward JW. *The snakes of Europe*. Newton Abbot: David and Charles Limited, 1971.

Steward MM. Affinity of the antibody-antigen reaction and its biological significance. In Glynn LE, Steward MW, eds. *Structure and function of antibodies*. Chichester: John Wiley and Sons, 1981, 233-59.

Sunthornandh P, Ratanabanangkoon K. A comparative study of three vehicles on antibody responses against elapid snake neurotoxin immunogens. *Toxicon* 1994;32:561-71.

Sutherland SK. Serum reactions. An analysis of commercial antivenoms and the possible role of anticomplementary activity in de-novo reactions to antivenoms and antitoxins. *Med J Aust* 1977;1:613-15.

Svendsen L, Crowley A, Ostergaard LH, Stodulski G, Hau J. Development and comparison of purification strategies for chicken antibodies from egg yolk. *Laboratory Animal Science* 1995;45:89-93.

Theakston RDG, Lloyd-Jones MJ, Reid HA. Micro-ELISA for detecting and assaying snake venom and venom-antibody. *Lancet* 1977;2:639-41.

Theakston RDG, Pugh RN, Reid HA. Enzyme-linked immunosorbent assay of venom-antibodies in human victims of snake bite. *J Trop Med Hyg* 1981;84:109-12(a).

Theakston RDG, Reid HA, Larrick JW, Kaplan J, Yost JA. Snake venom antibodies in Ecuadorian Indians. *J Trop Med Hyg* 1981;84:199-202(b).

Theakston RDG, Warrell DA, Griffiths E. Report of a WHO workshop on the standardization and control of antivenoms. *Toxicon* 2003;41:541-57.

Vandegaer JE, Reichmann ME, Cook WH. Preparation and molecular weight of lipovitellin from egg yolk. *Arch Biochem Biophys* 1956;62:328-37.

Von Behring E, Kitasato S. Ueber das Zustandekommen der Diphtherie-immunität und der Tetanus-immunität bei Thieren. *Dtsch Med Wochenschr* 1890;16:1113-1145.

Walsh K, Kauffman D, Sampath K, Neurath H. On the structure and function of bovine trypsinogen and trypsin. *Proc Nat Acad Sci* 1964;51:301-308.

Whitaker J. The sulfhydryl proteases: Food related enzymes. *Adv in Chem* 1974;136:202 - 208.

Wiedemann V, Linckh E, Kuhlmann R, Schmidt P, Losch U. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. V. In vivo studies on protective effects against *Escherichia coli* diarrhea in pigs. *Zentralbl Veterinarmed [B]* 1991;38:283-91.

Wiedemann V, Kuhlmann R, Schmidt P, Erhardt W, Losch U. Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. III. In vivo tenacity test in piglets with artificial jejunal fistula. *Zentralbl Veterinarmed [B]* 1990;37:163-72.

Williams J. Serum proteins and the livetins of hen's-egg Yolk. *Biochem J* 1962;83:346-55.

Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. *J Clin Invest* 1960;39: 1157-1175.

Zeller E. Enzymes of snake venoms and their biological significance. *Adv Enzymol* , 1948;8:459-95.